OSCAR KELLNER

(1851 – 1911)

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(May 13, 1851 — September 22, 1911)

It is generally accepted that German science in the period 1850 to 1914 was of very high standing. This was not least the case with chemistry and the related sciences, physiological chemistry and agricultural chemistry. Professor R. Adams Dutcher has said: "During this period Germany was a Mecca for foreign students seeking advanced training in the physiological and chemical sciences." As to standing in science, the position of Germany at that time reminds us much of that of the United States of America at the present time.

A high scientific standing is a result of work by numerous scientists, making their contributions both great and small. Some of them gain special distinction. Men may be especially gifted, born at the right moment, and with the golden ability to concentrate their efforts on their work. These great pioneers act as torchbearers leading the way for others. F. Honcamp has stated that in German agricultural chemistry there were three such pathfinders: Liebig, Hellriegel and Kellner.

Oscar Kellner was born in Tillowitz in Silesia on May 13, 1851. He went to the Realgymnasium (corresponding to the high school) at Neisse. In his last year of school he joined the Silesian corps as a volunteer and fought in the Franco-German war in 1870–1871. Shortly after the war Kellner finished school and went to the universities in Breslau and Leipzig to study the basic sciences, especially chemistry. As a student he had already acquired the diligence which was characteristic of his whole life, even by the standards of hard-working Germany. From the very beginning he felt impelled to do his best. This helps to explain how in the short space of three years he was able to get his Ph.D. degree at the University of Leipzig. His first scientific paper, about the chemistry of the sprouting of peas, published in 1874, was prepared when he was a student.

As a young doctor Kellner became an assistant in animal chemistry at the Agricultural Academy of Proskau (Silesia). The head of the institute was Professor Weiske, who had specialized in animal physiology and nutrition.

Kellner stayed in Proskau for only two years, but during this time he published 10 or 11 papers, alone or in cooperation with

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others. Besides his experimental work he gave lectures. One gets an impression of the growing esteem in which he was held from the fact that in 1875, at the age of 24, he gave a lecture at the 48th annual meeting of the German association of scientists and physicians. He spoke on how metabolism was influenced by removing the wool from sheep and by giving the sheep arsenic in their food.

In 1876 Kellner went to Hohenheim at Stuttgart (Würtemberg). Here he became an assistant to Professor Emil Wolff, who was of Danish origin. Before coming to Hohenheim, Wolff had been the first director (1851–1854) of the experiment station Möckern, at Leipzig, the first agricultural experiment station in Germany, erected in the year Kellner was born, and where he later was to find the main scene of his activities. When at Möckern, Wolff worked out his first tables on the composition of feeds. It was Wolff who first realized the importance of determining the digestibility of feeds. The Germans often preferred the term "Verwertung" (utilization) rather than "digestibility." That was because they found it necessary to emphasize that not the total amount of nutrients, but only the digested amounts, could be expected to have nutritive value. In the period about 1870 digestibility experiments and the determination of chemical composition were the two subjects on which the interest of animal nutritionists centered. The titles of the papers which Kellner published during the years at Proskau and Hohenheim show that already at that early period he was acquainted with the problems of feed evaluation, the field in which he later became the master.

In cooperation with Wolff he published 12 important papers. They dealt with experiments on various feeds for horses and ruminants. He also published the same number of papers alone. Both animal and plant chemistry are treated. One of these papers deserves special mention, because it cleared up an old controversial question in physiology — namely, what kind of nutrients are used in muscular work. On account of his great authority, Liebig's opinion that the muscles work by catabolizing protein — the material of which they are built — still prevailed. Accordingly, the nitrogen excretion in the urine would be expected to increase during muscular work. In a series of nicely conducted experiments with horses, published in 1879 and 1880, in which the work was measured by using a dynamometer, Kellner found that heavy work sometimes increased the nitrogen excretion, but not when sufficient amounts of carbohydrates were given. It was thus proved that nitrogen-free nutrients could be used as a source of energy in muscular work. It is noteworthy that Kellner already at that time indicated that the energy content of nutrients was perhaps the best measure of nutritive value. That happened three years before Rubner put forward his law of

isodynamic replacement, on which energy metabolism work later was based.

In plant physiology Kellner worked on the role of nitrogen in nutrition. The question of the importance of asparagin and the quantitative determination of various nitrogenous constituents were also treated by him.

The impression of versatility is confirmed by his work on the detoxification of lupines and on ensiling farm crops.

In addition to this comprehensive experimental work he also found time to give lectures. He thus spoke on certain aspects of protein metabolism both at the 50th and 52nd annual meetings of the above-mentioned association of scientists and physicians. In a few years his name was well known even outside Germany. In 1880, at the age of 29, Kellner was called to the Imperial University at Tokyo as a professor of agricultural chemistry. Here he was given a task worthy of his energy and abilities. Kellner stayed in Japan for 12 years.

It is not wrong to say that the foundation of agricultural chemistry in Japan was greatly influenced by Kellner. He first took charge of the teaching. It is not surprising that he stressed the importance of practical laboratory courses. He further took a leading part in the organization and reorganization of the experimental agencies in agriculture.

Very soon Kellner got important problems to work on. His all-around training was a great advantage because of the diversity of the problems. These were marked by the special conditions prevailing in Japan, and some of them were very different from those he had worked on in Germany.

It was necessary to obtain more knowledge of Japanese soils. Very important were the problems regarding the fertilization of rice. Kellner compared different phosphates and nitrogenous fertilizers. He showed that the action of the latter was influenced by the special climactic conditions.

The East Asiatic problem of utilizing human feces as fertilizers had also to be investigated. But Kellner did not forget his specialty, animal nutrition. He studied ensiling and the factors influencing ensiling losses. Methods for determining the nitrogen in silage were improved. The special Japanese feeds were also investigated, and he showed the importance of producing roughage of high quality by cutting the crops at earlier stages of development.

A very interesting problem which Kellner worked on in Japan was the chemistry of the development and nutrition of the silkworm. Here it may be mentioned that he showed that this organism produces fat from carbohydrates. The growing of rice and the produc-

tion of silk are both of great importance in Japan, and Kellner's work on these two fundamental industries was highly appreciated.

In Japan Kellner married a Japanese woman, and this fact, together with the success he had in his work, without doubt increased the country's appreciation of him. In 1892, however, he was called home to Germany to become director of the agricultural experiment station at Möckern. With this appointment began the period which has given the name of Kellner a special and lasting position in the science of animal nutrition.

As mentioned above, Möckern was the first agricultural experiment station in Germany. The directors of this station have been: Emil Wolff, 1851–1854; H. Ritthausen, 1854–1856; W. Knop, 1856–1866; Gustav Kühn, 1867–1892; Oscar Kellner, 1892–1911; and Gustav Fingerling, 1912–1944.

Gustav Kühn, who died suddenly in 1892 at the relatively young age of 53, after an operation on a foot, deserves to be mentioned here. It was he who laid the foundation for the admirable work done by Kellner. In 1879 Kühn built a well-functioning respiration apparatus for cattle, and trained an excellent staff. The accuracy with which these people were accustomed to work may be illustrated by the fact that scientifically educated chemists had to perform 100 trial analyses before they were allowed to carry out the same analysis in an actual experiment. Every detail in the respiration and metabolism experiments was worked through with similar accuracy and thoroughness. In this well-equipped laboratory Kellner now got an opportunity to work on feed evaluation, the problem which from his early twenties had interested him so much.

It appears that Kellner quite early in his career had the impression that the digestible nutrients were not a good measure of the nutritive value of feeds. The problem of evaluating protein, fat and carbohydrates taken together is difficult without knowledge of the *effect of each nutrient*. Another problem is that the effect of a certain quantity of digestible nutrients varies *with the kind of feed*. Accordingly, evaluation based on the digestible nutrients is rather unreliable, especially when feeding large amounts of roughage.

When Kellner took over the leadership at Möckern on February 15, 1893, he had first of all to prepare for publication the works of Kühn. This was completed after a year's work and comprised 580 pages.

Kühn in his experiments had determined nitrogen and carbon balances in cattle, and thus was able to calculate the protein and fat retained. Kellner, following up his own ideas from 1880, introduced calorimetry, thus placing feed evaluation on an energy basis.

The effects of various pure nutrients — starch, cellulose, sugar, protein and fat — were studied by determining the amount of fat in the body produced from each nutrient. That was the birth of the "net energy" concept, which is based on the idea that nutrients and feeds should be evaluated on the basis of the effect produced upon the animal.

In this way it became possible for Kellner to evaluate the three main groups of digestible nutrients on a net energy basis. The next step was to determine the value of the digestible nutrients as present in feeds compared with that for the pure nutrients themselves. Kellner studied no less than 51 samples of 37 feeds systematically chosen from the most important feed groups. This list has been considerably extended by his successor, Fingerling.

Certain feeds, especially concentrates, had a productive value closely corresponding (95–100%) to the values that could be calculated from the content of digestible nutrients. Other feeds, such as fibrous roughages, showed net energy values far below the calculated values. After 12 years of work, Kellner felt he had sufficient material on which to base a system of feed evaluation. This was published in the first edition of his book, "Ernährung der landwirtschaftlichen Nutztiere," in 1905. This book came out in 6 editions from 1905 to 1912 and further editions were published later (the 10th in 1924). A smaller book, "Grundzüge der Fütterungslehre," which in 1940 reached its 9th edition, was also helpful in spreading knowledge of his system.

The *starch equivalent* — the unit introduced as a measure of the net energy of feeds in the fattening of adult cattle — was used directly or in modified form throughout Europe, and in a few years fully replaced "digestible nutrients." The starch equivalents are *calculated on the basis of the digestible nutrients* by using Kellner's factors for the net energy of the various nutrients and his "value numbers" for the different feeds. Instead of value numbers, "fiber reduction" is used for roughages. Kellner thus linked together the old and the new points of view, giving a fine example of the continuous development which characterizes true progress.

It has been claimed that Kellner's work has only historical interest. Others, by statistical calculations, have thrown doubt on his system and the scientific basis for it. These statements are, however, the results of misunderstandings and misinterpretations. Kellner's system of feed evaluation is still used, and evidence of its validity has been produced even in recent years. In England Yeates and coworkers in 1942 found the starch equivalents superior to other systems of feed evaluation. In 1949 in Switzerland the same was very convincingly stated by Crasemann and Schürch. In 1950 in

Sweden Olsson, and in Finland Poijärvi, demonstrated the superiority of "net energy," calculated after a modified method of Kellner's, over "metabolizable energy." Very recently Saarinen, Shaw, Sami, Moore and Irvin in the United States have found that the effect of concentrates added to roughage may be explained on the net energy basis. Here too the net energy was calculated by Kellner's method.

Thus, Kellner's work seems to be of some permanent value, which he himself, not very modestly, prophesied. How can this be explained? The answer is simple. Kellner attacked the problems with the chemist's sense of accurate quantitative determinations. The chemist of the turn of the century did not have so many different substances to work on as is the case today. The methods used by those studying energy metabolism were very accurate, even judged by present standards. Another sound chemical principle, namely, that experimental results are reliable only if they can be reproduced, was strongly stressed by Kellner. The planning of his experiments showed, however, that Kellner mastered not only chemistry but also physiology. It is tempting to say that he had physiological intuition.

He stressed the importance of working systematically. When the aim was to determine the effects of nutrients and feeds, it was necessary to begin with the simplest conditions. He therefore chose fat production in cattle. That this was a good choice was many years later confirmed in Denmark by Møllgaard.

About 1930 it was shown that the net energy of feed rations and single feeds was influenced by the composition and make-up of the rations. It was then said that the net energy values of single feeds were unreliable. But, as H. H. Mitchell stated in 1937, the nutritive values are significant if the feeds are used in *balanced rations,* and that was indeed the case with Kellner's rations.

It is very interesting to note that even before 1900 Kellner clearly recognized the importance of using balanced rations. He established border values, within which the protein amounts were allowed to vary. The basal rations, to which the nutrients and feeds were added, consisted of hay plus two or three kinds of concentrates, certainly a balanced ration for full-grown ruminants, especially as it was stressed that the hay should be cut early and be of uniform quality. That fermentation in the rumen may sometimes have a disturbing influence was also recognized by Kellner. He thus found that 63% of the metabolizable energy in starch was net energy, when starch was added to a balanced ration of hay and concentrates. Kühn and later Armsby found only 50% net energy when starch was added to hay alone. In the latter case the fermentation losses were much higher.

The introduction of the "starch equivalent" and the experimental work on which this is based stand in the foreground of what we owe to Kellner. But during his career in Möckern he also worked on other problems, mostly feeding problems. He carried out experiments which increased our knowledge about different feeds. Experiments on milk production and on the minerals calcium and phosphorus were conducted. He took the initiative to secure cooperative effort between different experimental stations, and some important topics were investigated in this fashion. They involved experiments on milk cows, horses and swine. One of the problems was to determine the extent to which corn can be replaced by potatoes, "the European corn." Some experiments on values of fertilizers were also carried out.

As to the work he did and his ability to work, nothing surprises us concerning Kellner. In the last years of his life his health was declining, but his love of work was unimpaired. Until the very last he had ideas that pressed him on. In the summer of 1911 he participated in the Dairy Congress at Stockholm, and at this meeting he gave one of the main lectures, based on material from new experiments on energy metabolism in dairy cows. The aim was evidently to extend the applicability of his system of feed evaluation. It was left to Møllgaard, however, to clear up this problem, following up the ideas of Kellner. At a meeting of the association of German agricultural experimental stations in the autumn of 1911 Kellner died from a heart attack, at 60 years of age, thus passing his unsolved problems on to others. When Fingerling became the director at Möckern in 1912, the distinction of Kellner's name secured liberal funds for him. By building many new respiration apparatuses that operated more automatically than the old ones, Fingerling was able to perform metabolism experiments on what amounted to a mass scale. With the exception of bran, for which he found a higher value, he confirmed all of Kellner's net energy determinations, thus confirming the view that the system of Kellner has a safe foundation.

The well-equipped laboratory in Möckern is still able to function. It was not destroyed during the war. But unfortunately it is on the wrong side of the iron curtain, and it seems that for unknown reasons the distinguished work of Kühn, Kellner and Fingerling is not being continued. It appears that quite a large number of the experiments performed by the two last scientists have not been published.

In the last 10 years of his life Kellner received numerous honors. He had the happy fate to see that his work was acknowledged while he lived. He received the title of Geh. Hofrat, Professor (Honorary), from Tokyo University, an honorary doctorate of medicine from Breslau University, and was awarded the Liebig medal in gold; many other orders of honor were also given him. Kellner was elected

an honorary member of many associations, and chairman of others. In addition, he was editor of "Biedermanns Centralblatt für Agrikulturchemie" and "Die Landwirtschaftlichen Versuchsstationen."

How can it be explained that one man accomplished as much as Kellner did? His love of work, and ability to concentrate on it, his rich personal gifts and never-tiring interest are some of the reasons for his success. In his later years, with his ever-increasing official tasks, he spent his Saturdays and Sundays in preparing the experimental material collected by his co-workers. Seen against this background, it does not seem strange that in the later years he was not able to publish reports on larger projects. Many of them, however, were subsequently published by his successor, Fingerling. The somewhat authoritative system of Germany perhaps makes it possible for a man with the gifts of Kellner to contribute so much. Now, however, we find it remarkable that his co-workers seemed to exist mainly to carry out his orders. Everything seems to have been concentrated in Kellner's brain.

Kellner was endowed with a strong personality; he was not afraid of a fight. This does not appear to be at all unusual in people working on energy metabolism and feed evaluation in Europe, as in the United States. It may be that working on these problems demands a certain combative character if great results are to be attained. Here, perhaps, is one of the keys to Kellner's success.

The motto of Kellner was, "Es geht alles, man muss nur wollen" (Everything is possible, it is only a question of will). He demanded much of himself and of his co-workers as well. It is said that his first assistant, Professor Köhler, began his work at 4:30 A.M. in order to get everything ready for the respiration experiments which were started at 8:00 A.M. Kellner appreciated good work and was a good man to work with. The high morale of the Möckern station, still easily recognizable when I worked there for a while nearly 30 years after his death, seems to one who did not have the privilege of knowing Kellner personally to be something very largely his creation. The institution was a reflection of the man.

KNUT BREIREM
Royal Agricultural College of Norway
Division of Animal Nutrition
Vollebekk

THE EFFECTS OF DIET, PARTIAL HEPATECTOMY AND GROWTH-PROMOTING FACTORS ON THE COMPOSITION OF THE RAT LIVER [1]

MARY E. DUMM, ELAINE P. RALLI, HERBERT GERSHBERG AND BERTRAM LAKEN

Department of Medicine, College of Medicine, New York University-Bellevue Medical Center, New York, N. Y.

THREE FIGURES

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INTRODUCTION

The effectiveness of therapy in liver damage has generally been related to the lipotropic activity of the agent used. The lipotropic effects of choline, methionine and liver extract have been reported by numerous investigators (Best et al., '33, '50; Hall and Drill, '48; György and Goldblatt, '49; Tucker and Eckstein, '37; Channon et al., '38). Conflicting reports have appeared on the lipotropic effect of vitamin B_{12} (Drill and McCormick, '49; McCormick and Drill, '50; György and Rose, '50; Lucas et al., '51). In the majority of experimental studies, liver damage has been produced in rats by the feeding of a low protein-high fat diet. Although this diet does result in an increased lipid content of the liver, the degree of fatty infiltration varies considerably among the individual animals.

In the studies we are reporting a low protein-high fat diet was used and the effect of the liver damage was intensified by removing approximately 70% of the liver after a period of 35 to 42 days on the diet. This procedure also made it possible for each animal to serve as its own control.

[1] This research was aided by grants from the U. S. Public Health Service and the Lederle Laboratories.

EXPERIMENTAL PROCEDURES

Seventy-five-day-old male rats, from our own colony, were placed on a diet containing 36% hydrogenated cottonseed oil,[2] 9% vitamin-free casein,[3] 48% sucrose, 2% cod liver oil and 5% salt mixture.[4] The diet was supplemented as follows, in milligrams per 100 gm: thiamine 1, pyridoxine 1, riboflavin 2, pteroylglutamic acid 0.2, biotin 0.05, calcium pantothenate 1, niacin 10, mixed tocopherols 15, vitamin K[5] 15. After approximately 42 days on this diet, the animals were partially hepatectomized by the dorsal route, as previously described (Ralli and Dumm, '51). Following surgical removal of approximately 70% of the liver, some animals were continued on the low protein-high fat diet. Others were transferred either to a high protein diet (26% casein, 7% hydrogenated cottonseed oil, 60% sucrose) or to a moderate protein-low fat diet (16% casein, 7% cottonseed oil, 70% sucrose). The vitamin supplements were the same in all the diets.

The effect of supplementing the low protein-high fat diet with liver extract[6] (0.06 ml daily) or with vitamin B_{12}[7] (5 μg daily) was studied. These substances were injected subcutaneously. Since the liver extract used contained 2.4 μg of vitamin B_{12} per milliliter, the rats on liver extract received 0.004 μg of vitamin B_{12} daily as contrasted with 5 μg daily of the vitamin B_{12} concentrate. The control animals received an equal volume of 0.9% saline, subcutaneously. Therapy was continued from 5 to 14 days after partial hepatectomy, at which time the animals were sacrificed. Evaluation of the changes in the composition of the liver after therapy was made by determining the solids, total lipids and nitrogen con-

[2] Primex.

[3] Borden's.

[4] The salt mixture consisted of ferric citrate 2%, calcium phosphate (dibasic) 44%, potassium citrate 42%, magnesium citrate 12%, copper sulfate 0.09%, potassium iodide 0.005%. All rats received 1% NaCl as drinking water.

[5] Hoffmann-La Roche "Synkavit"- 2-methylnaphthohydroquinone-1,4-tetra sodium diphosphate.

[6] Lederle's Intraheptol.

[7] Lederle's B_{12} concentrate.

tent of the liver removed at the time of partial hepatectomy and at the time of sacrifice.

Solids were determined by drying the liver tissue for 72 hours in an oven at 95° to 97°C., total lipids by extraction of minced liver tissue as previously described (Rubin et al., '37), and nitrogen by micro-Kjeldahl digestion, followed by direct nesslerization of a suitable aliquot of the neutralized digest. Samples of liver for microscopic examination were taken at the time of operation and again at the time of sacrifice, in each animal.

The livers of intact rats on a normal diet (22% protein) with 30 mg of choline per 100 gm of diet and on the same diet without choline were also analyzed. Similar analyses were done on intact rats after varying periods on the low protein-high fat diet, in order to determine the effect of the duration of the low protein diet on the liver constituents. The animals were weighed at frequent intervals during the course of the experiments. Food consumption was determined in a series of animals on each diet studied.

RESULTS

Intact rats

All results to be presented here are given in terms of the wet weight of the liver.

The results on the intact rats are given in table 1. On the normal diet with added choline, the liver lipids were lower than on this diet without choline. According to Best et al. ('50), more than 30 mg of choline per 100 gm of diet are required to obtain the maximum lipotropic effect in rats on a diet low in protein. The results of our experiments indicate that small doses of choline will reduce the concentration of liver lipids in rats on a normal protein intake. The liver nitrogen in rats on the diet with added choline was 3.65 ± 0.06 gm %, which is similar to 3.51 ± 0.26 gm %, the mean liver nitrogen reported by Brues et al. ('36). On the diet without added choline the liver nitrogens

TABLE 1

Effect of duration of low protein-high fat diet [1] *on liver constituents of intact rats compared to the effect of "normal" diets*

DIET	DAYS ON HIGH FAT DIET	NUMBER OF RATS	TOTAL FAT	TOTAL NITROGEN	NITROGEN	TOTAL SOLIDS	FAT-FREE SOLIDS
			gm% ± S.E.	*gm% ± S.E.*	*× 6.25 ± S.E.*	*gm% ± S.E.*	*gm% ± S.E.*
Normal diet [2]							
With choline	None	6	0.76 ± 0.08	3.65 ± 0.06	22.8 ± 0.37	28.4 ± 0.31	27.7 ± 0.29
Without choline	None	4	2.55 ± 0.41	3.17 ± 0.15	19.8 ± 0.98	30.3 ± 0.16	27.5 ± 0.38
Low protein-high [1] fat diet	22	10	10.1 ± 0.93	2.90 ± 0.11	18.1 ± 0.69	32.8 ± 0.89	22.7 ± 0.87
	30	8	8.3 ± 1.65	3.25 ± 0.03	20.3 ± 0.19	33.0 ± 1.90	24.7 ± 1.33
	38–44	46	11.1 ± 0.77	3.05 ± 0.05	19.1 ± 0.31	34.8 ± 0.76	23.6 ± 0.48
	51–59	7	6.3 ± 1.33	2.79 ± 0.19	17.4 ± 1.18	33.8 ± 1.75	27.6 ± 0.51

[1] Composition of low protein-high fat diet: protein 9%; fat 38%; carbohydrate 48%; salts 5%.

[2] Composition of normal control diet: protein 22%; fat 11%; carbohydrate 62%; salts 5% — with 30 mg of choline per 100 gm of diet, or without choline. These were our colony rats that had been on dog chow from the time of weaning until 80 days of age. They were transferred to a "normal" control diet and kept on this for 5 days.

were lower than on the diet with choline. After a period of 22 days on the low protein-high fat diet, the concentration of fat increased in the livers of the intact rats. This was associated with a decrease in liver nitrogen. Analyses done after 51 to 59 days on this diet showed a decrease in liver lipids, probably associated with the development of fibrosis in the liver. The liver lipids were still higher than the values observed in normal rats.

Partially hepatectomized rats

Following partial hepatectomy, all of the rats lost weight regardless of the post-operative nutritional therapy. The weight changes for a typical rat in each group are shown in figure 1. On the low protein-high fat diet the rats continued to lose weight for 7 to 9 days after partial hepatectomy. By the 10th day they were generally gaining weight, but only occasionally had reached their operative weight by the time of sacrifice. On the moderate protein diet the rats lost weight during the first 5 to 7 days after partial hepatectomy and by 12 to 14 days their weights usually approached the weight at operation. The animals on the high protein diet began to regain weight by the second post-operative day, and by the 7th day had generally reached or surpassed their weight at operation. It was therefore felt that by the 10th day after partial hepatectomy the lipotropic effects of the nutritional supplements tested were not masked by the catabolic processes.

The food intake of a series of rats on each diet studied was measured. Intact rats on the normal diet consumed approximately 12 to 14 gm of food daily, which provided 50 to 60 cal. and about 3 gm of protein. On the low protein-high fat diet, the daily food intake of the intact rats before hepatectomy was usually from 9 to 11 gm, which supplied 50 to 60 cal., but only about 1 gm of protein. Following partial hepatectomy, the food intake for all groups of animals was low. By the third day after partial hepatectomy, the rats

transferred to the high protein diet were eating well, and from three to 7 days after partial hepatectomy consumed from 11 to 12 gm of the diet daily, which provided about 50 cal. and about 3 gm of protein. All groups of rats continued on the low protein-high fat diet ate poorly until 5 to 8 days

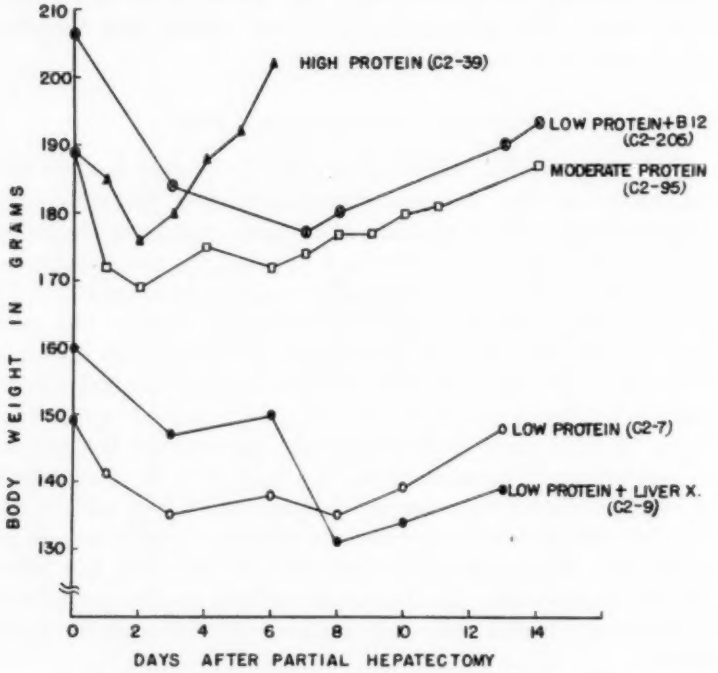


Fig. 1 Weight changes in rats following partial hepatectomy.

after the operation. Thereafter, the rats receiving the diet alone, and those given, in addition, liver extract, ate from 6 to 9 gm of diet daily, which supplied them with from 35 to 50 cal. and from 0.6 to 0.8 gm of protein. By 8 to 14 days after partial hepatectomy, the rats on the low protein-high fat diet supplemented with vitamin B_{12} had returned to approximately their pre-operative food consumption of 9

to 11 gm daily. Animals whose food consumption following partial hepatectomy did not improve usually died during the first week.

The data on the partially hepatectomized rats continued on the low protein-high fat diet and receiving no additional supplement after partial hepatectomy are given in table 2. Comparison of the liver constituents at partial hepatectomy and at sacrifice showed an increase in liver lipids in all but two animals in this group. The concentration in liver lipids at the time of partial hepatectomy varied from 3.5 to 19.5 gm % and at sacrifice from 3.4 to 25.2 gm %. These results illustrate the variations in liver lipid concentrations that will occur in individual animals treated in a similar manner and maintained on a low protein-high fat diet. Variation in the nitrogen content of the liver was less than in the lipid content; i.e., 2.2 to 3.7 gm % at partial hepatectomy and 2.3 to 3.4 gm % at sacrifice. Nitrogen decreased in the livers of 5 of the 7 rats from the time of operation to the time of sacrifice.

When the rats were given liver extract or vitamin B_{12} after partial hepatectomy, distinct changes occurred in the lipid concentration in the liver. The results are given in table 3. In all of the animals the liver lipids had decreased at the time of sacrifice. The average liver lipid content in the rats receiving liver extract was 7.3 gm % at the time of sacrifice, an average decrease of approximately 5 gm %. In the rats receiving vitamin B_{12}, the average liver lipid concentration was 6 gm % at sacrifice, which represented an average decrease in lipid concentration of approximately 8 gm %. The results indicate that both liver extract and vitamin B_{12} had a lipotropic effect, in spite of the fact that the animals were continued on a low protein-high fat diet and that 70% of the liver had been removed. In the rats receiving vitamin B_{12}, the liver lipids had reached normal levels in three animals at the time of sacrifice. In 4 animals the concentration of nitrogen in the liver had increased.

TABLE 2

Data on control rats continued on low protein-high fat diet[1] after hepatectomy with no additional supplement

RAT NO.	DAYS ON DIET PRE-HEPATECTOMY	WEIGHT OF RAT AT OPERATION	DAYS POST-HEPATECTOMY	WEIGHT OF RAT AT SACRIFICE	ANALYSES OF LIVER								
					At partial hepatectomy				At sacrifice				
					Gm of liver removed	% Lipids	% Nitrogen	% Solids	Weight of liver at sacrifice	% Lipids	% Nitrogen	% Solids	% Liver weight restored
		gm		*gm*					*gm*				
C2-18	38	155	6	137	5.25	15.1	2.45	34.6	5.18	12.1	2.85	...	58
C2-236	30	218	7	208	7.23	15.6	3.30	45.5	9.30	18.8	2.73	41.7	88
C2-227	42	137	12	127	4.27	3.5	...	30.0	5.04	3.4	3.35	26.3	77
C2-228	42	170	12	149	5.03	13.5	3.72	37.2	5.93	20.2	3.10	39.5	77
C2-229	42	195	12	198	6.51	19.5	3.32	40.5	8.90	25.2	3.03	47.6	95
C2-7	22	149	14	148	4.51	15.0	2.21	35.1	8.14	24.4	2.33	43.6	139
C2-10	22	154	14	158	3.43	11.4	3.29	31.0	6.45	20.6	2.60	36.7	148
C2-2	22	166	15	142	3.64	6.1	2.98	30.5	7.23	7.4	2.76	28.0	158
C2-4	22	126	15	122	3.83	7.6	3.01	32.0	4.62	16.2	...	38.5	80
Average						11.9	3.04	35.2		16.5	2.77	37.7	

[1] Composition of low protein-high fat diet: protein 9%; fat 38%; carbohydrate 48%; salts 5%.

TABLE 3

Liver constituents in rats on low protein-high fat diet [1] before and after partial hepatectomy plus either liver extract or B_{12} after operation

RAT NO.	DAYS ON DIET PRE-HEPAT-ECTOMY	WEIGHT OF RAT AT OP-ERATION	DAYS POST-HEPAT-ECTOMY	WEIGHT OF RAT AT SAC-RIFICE	DIET SUPPLE-MENT POST-HEPAT-ECTOMY	ANALYSES OF LIVER								
						At partial hepatectomy				At sacrifice				
						Gm of liver removed	% Lipids	% Nitrogen	% Solids	Weight of liver at sacrifice	% Lipids	% Nitrogen	% Solids	% Liver weight restored
		gm		gm						gm				
C2-19	38	151	6	137	L.E.	5.26	15.6	2.46	34.5	6.30	12.4	2.62	33.2	79
C2-30	38	169	8	145	L.E.	4.58	12.9	3.27	33.2	6.23	8.0	...	29.3	95
C2-9	22	160	14	139	L.E.	4.50	10.0	3.23	31.8	4.56	3.1	2.85	24.3	60
C2-3	22	144	15	134	L.E.	3.53	10.0	2.77	29.7	5.93	5.7	2.89	27.6	127
Average							12.1	2.82	32.3		7.3	2.79	28.6	
C2-201	44	155	7	137	B_{12}	5.53	20.9	2.78	42.7	3.28	7.4	3.18	29.2	18
C2-202	44	218	7	207	B_{12}	9.53	19.4	2.61	52.1	9.42	12.8	2.35	39.3	58
C2-224	42	178	12	140	B_{12}	6.13	10.8	2.99	34.6	6.63	1.5	3.42	23.4	67
C2-203	44	194	14	218	B_{12}	5.09	7.2	3.20	34.1	5.69	2.1	3.35	33.0	71
C2-204	44	215	14	240	B_{12}	5.90	..	2.82	34.0	7.30	2.0	3.27	30.7	83
C2-205	44	206	14	193	B_{12}	7.66	12.0	2.90	45.5	5.91	6.1	2.94	32.9	36
Average							14.1	2.88	40.5		6.0	3.05	31.4	

[1] Composition of low protein-high fat diet: protein 9%; fat 38%; carbohydrate 48%; salts 5%.

In two groups of animals, following partial hepatectomy, a change of diet was instituted in order to compare the results with those obtained when the growth-promoting factors were fed. The data are given in table 4. Eight animals were placed on a moderate protein-low fat diet following partial hepatectomy (protein 16%, fat 9%, carbohydrate 70%) and 4 animals were given a high protein diet (protein 26%, fat 9%, carbohydrate 60%). On the moderate protein diet, at the time of sacrifice the liver lipid concentration had decreased in 4 animals. In two animals there was a slight increase in the liver lipids and in two animals the lipid determinations of the liver were lost at the time of sacrifice. In the animals receiving the high protein diet, the lipid concentration was decreased in all 4 animals at the time of sacrifice. The per cent nitrogen in the liver at the time of sacrifice had increased in all of the animals changed to the moderate or high protein diet.

The per cent of liver weight restored following partial hepatectomy is given in the last column of tables 2, 3 and 4. This was calculated on the basis of the known relationship of the individual liver lobes to the total liver weight. It was previously reported (Ralli and Dumm, '51) that after 6 weeks on the low protein-high fat diet, the median and left lateral lobes of the liver averaged 71% of the total liver weight. This is approximately the relationship that is found in normal rat livers. Following partial hepatectomy in the rats continued on the low protein-high fat diet for 12 days or more and receiving no additional supplements (table 2), the per cent of the liver weight restored varied from 77 to 158. The calculation of liver weight restored obviously does not reflect the nature of the tissue, and if used as the sole index of the regeneration of tissue, could be misleading. For example, in the rats continued on the low protein-high fat diet with no supplement, chemical analyses and microscopic sections of the livers demonstrated clearly that the weight of the livers was due in part to the accumulation of fat. If one contrasts this with the per cent of liver re-

TABLE 4

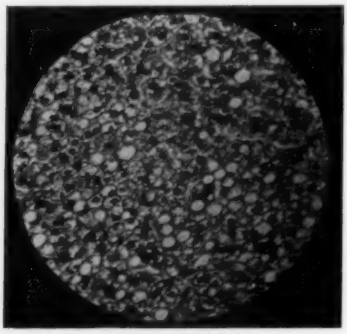
Liver constituents in rats on low protein-high fat diet before and on moderate[1] or high-protein[2] diet after partial hepatectomy

RAT NO.	DAYS ON DIET PRE-HEPAT-ECTOMY	WEIGHT OF RAT AT OP-ERATION	DAYS POST-HEPAT-ECTOMY	WEIGHT OF RAT AT SACRI-FICE	POST-HEPAT-ECTOMY DIET	ANALYSES OF LIVER								
						At partial hepatectomy				At sacrifice				
						Gm of liver removed	% Lipids	% Nitrogen	% Solids	Weight of liver at sacrifice	% Lipids	% Nitrogen	% Solids	% Liver weight restored
		gm		*gm*						*gm*				
C2-72	38	226	4	234	Moderate protein	5.66	7.2	...	33.7	6.28	9.6	3.41	28.4	70
C2-68	38	174	7	167		4.03	6.8	3.06	29.8	7.01	..	3.65	23.9	132
C2-67	38	156	7	166		3.58	9.3	3.66	27.8	5.61	..	3.59	33.5	115
C2-54	39	187	7	176		6.79	18.2	2.86	39.1	8.44	8.2	3.10	35.9	84
C2-92	43	175	15	142		6.93	21.8	2.68	45.0	6.86	4.9	3.57	23.3	58
C2-95	43	189	15	188		6.35	11.8	2.87	40.5	7.12	8.3	3.50	33.1	71
C2-97	44	192	15	180		4.65	5.3	3.32	30.9	5.07	4.2	4.12	29.2	68
C2-101	44	176	15	191		4.38	7.8	2.94	31.9	5.70	10.7	3.62	33.9	92
Average							12.0	3.06	34.8		7.6	3.38	30.2	
C2-36	38	187	5	185	High protein	7.56	25.5	2.42	46.3	7.97	12.6	3.09	32.1	64
C2-39	38	189	7	202		5.84	13.2	2.67	31.9	6.40	5.9	3.29	29.4	71
C2-43	30	165	7	182		4.95	14.7	...	...	7.48	5.1	...	...	110
C2-47	42	185	7	190		4.63	12.1	2.68	31.9	7.89	4.4	3.37	29.3	129
Average							16.4	2.59	36.7		7.0	3.25	30.3	

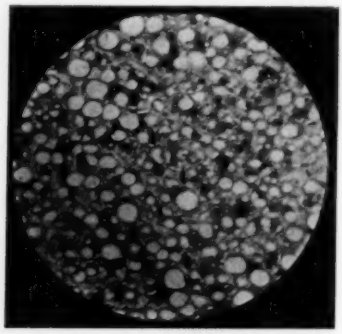
[1] Composition of moderate protein diet: protein 16%; fat 9%; carbohydrate 70%; salts 5%.
[2] Composition of high protein diet: protein 26%; fat 9%; carbohydrate 60%; salts 5%.

stored following the administration of vitamin B_{12} or liver extract (table 3), it is clear that the decrease in lipids, which is a desirable therapeutic effect, was usually associated with a smaller restoration of liver weight. The per cent of liver weight restored was in general greater in the animals changed to the moderate or high protein diet (table 4) than in the animals receiving liver extract or vitamin B_{12}.

A. Microscopic sections from liver of control rat on high fat diet before and after partial hepatectomy.

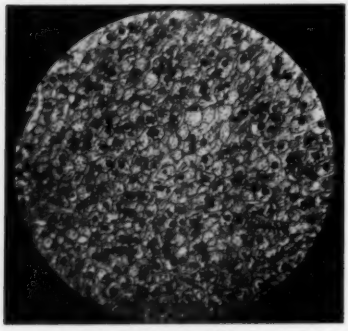


AT HEPATECTOMY
RAT C2-236

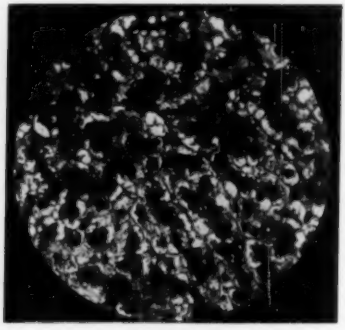


AT SACRIFICE
7 DAYS LATER

B. Sections from liver of rat on high fat diet plus liver extract after partial hepatectomy.



AT HEPATECTOMY
RAT C2-30



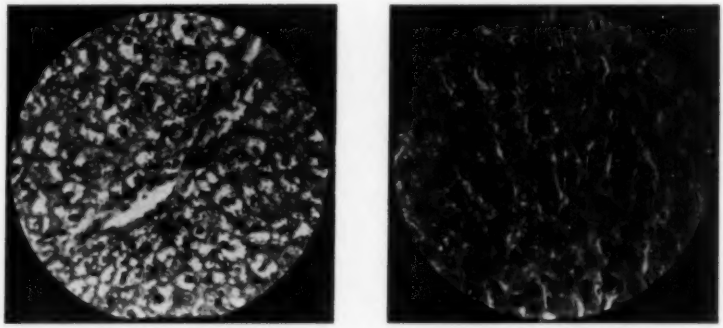
AT SACRIFICE
8 DAYS LATER

Figure 2

Microscopic appearance of the livers

The microscopic appearance of the livers is shown in figures 2 and 3. Figure 2 A illustrates the microscopic appearance of the liver at the time of partial hepatectomy and at the time of sacrifice of a rat (C2-236) continued for 12 days on the low protein-high fat diet following partial hepatectomy and receiving no nutritional supplement. In this

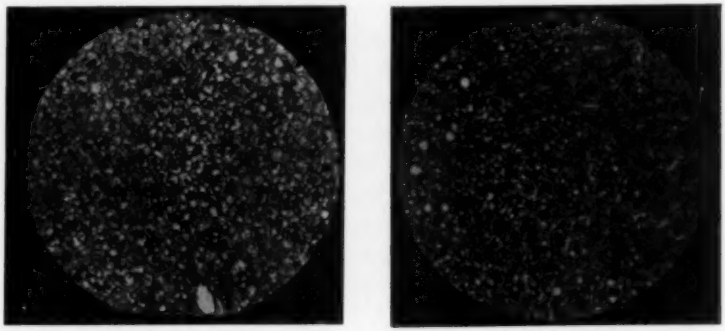
A. Sections from liver of rat on high fat diet plus B_{12} after partial hepatectomy.



AT HEPATECTOMY
RAT C2-224

AT SACRIFICE
12 DAYS LATER

B. Sections from liver of rat changed to high protein diet after partial hepatectomy.



AT HEPATECTOMY
RAT C2-43

AT SACRIFICE
7 DAYS LATER

Figure 3

animal at the time of operation the total liver lipids were 19.5 gm % and at sacrifice the lipids had increased to 25.5 gm %. Microscopically, at the time of partial hepatectomy there was evidence of diffuse fatty infiltration and 12 days later, when the animal was sacrificed, both the degree and area of fatty infiltration of the liver had increased and destruction of liver cells with beginning fibrosis was observed.

Figure 2 B shows sections from a rat (C2-30), also continued on the low protein-high fat diet after partial hepatectomy, which was given, in addition, liver extract. In this rat the liver lipids at the time of partial hepatectomy were 12.9 gm %. The animal was sacrificed 8 days later, having received injections of liver extract daily. At the time of sacrifice the liver lipids were 8 gm % and there was definite evidence of stimulation to regeneration of liver cells. Microscopically, fat deposition had decreased in the liver and the staining capacity of the cells had improved.

In figure 3 A are shown microscopic sections from the liver of a rat (C2-224) on the low protein-high fat diet which received vitamin B_{12} following partial hepatectomy. Again, at operation, microscopic evidence of fatty infiltration and destruction of liver cells is evident. Following 12 days of vitamin B_{12} therapy, the liver showed marked cellular regeneration and a striking decrease in the amount of fat. The liver lipids fell from 10.8 gm % to 1.5 gm %. These microscopic sections are characteristic of the response in the animals receiving vitamin B_{12}, and the increase in the staining reaction of the cells was one of the most striking findings.

Figure 3 B shows the microscopic sections of a rat (C2-43) changed to the high protein diet but receiving no additional nutritional supplement. In this rat the lipid content of the liver decreased and, microscopically, evidence of regeneration of liver tissue was present but was actually not as marked as in the rats receiving either vitamin B_{12} or liver extract on the low protein-high fat diet.

All enlargements are $\times$ 350. The microscopic sections of the rats receiving vitamin B_{12} or liver extract give the im-

pression of a somewhat greater enlargement. This is apparently due to the fact that, microscopically, following the administration of these two supplements, the liver cells and cords were increased in size.

DISCUSSION

The most striking changes in the composition of the liver in the animals in this study occurred in the rats treated with vitamin B_{12} following partial hepatectomy, even though they were continued on a low protein-high fat diet. A decrease in liver lipids, an increase in liver nitrogen and an improvement in the staining reaction of the cells were characteristic. Increasing the protein content of the diet following partial hepatectomy also exerted a lipotropic effect and improved the condition of the liver cells. This result on the high protein diet has been reported both experimentally and clinically (Lowry et al., '41; Patek and Post, '41; György and Goldblatt, '49).

Although the role of vitamin B_{12} in intermediary metabolism is not finally established, many investigators have suggested that this vitamin is implicated in the synthesis and transfer of labile methyl groups. Young rats and chicks fed diets deficient in labile methyl groups, but containing folic acid, will grow if adequate amounts of vitamin B_{12} are given (Schaefer et al., '49, '50b; Stekol and Weiss, '50; Bennett, '50; Stokstad et al., '50; Salmon, '50). The lipotropic effect of vitamin B_{12} in the rat and chick has been attributed to its role in methylation processes (Schaefer et al., '50a; Bennett et al., '51). Vitamin B_{12} has also been implicated in the synthesis of nucleic acids in bacteria (Shive et al., '48a; Wright et al., '48; Shive et al., '48b). Many investigators consider that the concentration of pentose nucleic acids is related to the degree of protein synthesis (Caspersson, '39, '47; Brachet, '47, '50; Spiegelman, '46). Changes in the nucleic acid content of the liver have been reported in experimental situations associated with disturbances in protein metabolism (Drabkin, '47; Novikoff and Potter, '48). Weser

and Popper ('49) found that the loss of pentose nucleic acid which usually occurs following liver damage induced by carbon tetrachloride was prevented by the administration of vitamin B_{12}. The improved staining reaction of liver cells observed during liver regeneration (Brachet et al., '46; Novikoff and Potter, '48) is believed to be due to an increase in pentose nucleic acid.

In the studies we are reporting, the lipotropic effect of vitamin B_{12} is probably related to the role of this vitamin in the synthesis and transfer of labile methyl groups. The observed increase in the protein content of the liver and the improvement in the staining properties of the liver cells are consistent with the suggestion that vitamin B_{12} may also have some role in the metabolism of the nucleic acids. In our experiments, vitamin B_{12} was more effective in improving the condition of the damaged liver than was either liver extract or an increase in the protein content of the diet.

SUMMARY

The effect of normal diets (22% protein) and of low protein-high fat diets (9% protein) on the liver constituents of intact rats was studied. The liver lipids in intact rats on normal diets were lower when 30 mg of choline were added daily to the diet than on this diet without choline (0.76 gm % as compared to 2.55 gm %). In intact rats on the 9% protein diet the lipid content of the liver varied, averaging about 10 gm % after 22 days on the diet. The increase in liver lipids was associated with a decrease in the nitrogen content of the liver.

After being maintained on the low protein-high fat diet for about 42 days, groups of rats were partially hepatectomized. When they were continued on this diet, the lipid content of the liver was further increased at the time of sacrifice, 6 to 15 days later. When the rats continued on the low protein-high fat diet after partial hepatectomy were given 5 μg of vitamin B_{12} by injection daily, there was a consistent decrease in the liver lipids and an increase in liver nitrogen at the

time of sacrifice. The injection of liver extract was also associated with a decrease in the liver lipids, but not as consistent a decrease as that occurring with vitamin B_{12}. Increasing the protein content of the diet to 16% following partial hepatectomy was associated with some lipotropic effect and an increase in the liver nitrogen, but this was not as uniform as when the protein in the diet was increased to 26%.

Microscopic examination of the livers of the rats at the time of hepatectomy and at the time of sacrifice showed an increase in lipid deposition in the animals continued on the low protein diet, and not receiving any additional therapy. The administration of both liver extract and vitamin B_{12} was associated with microscopic evidence of regeneration of liver cells, more marked in the animals receiving vitamin B_{12}. In the animals changed to the 26% protein diet, regeneration of the liver cells was present but to a less marked degree.

ACKNOWLEDGMENTS

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THE PROTECTIVE EFFECT OF DIETARY FAT ON IMMATURE RATS FED THYROID [1]

SAMUEL M. GREENBERG [2]

(WITH THE TECHNICAL ASSISTANCE OF LYDIA P. L. CHEN AND MARILYN A. BINGEMANN)

Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

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The responses of animals to thyroid show marked differences according to the dietary components which are fed concomitantly. Nichol et al. ('49), Emerson ('49), and Ershoff ('49) showed that, on diets containing soybean meal as a protein source, growth retardation of thyroid-fed rats and chicks is counteracted by crystalline vitamin B_{12}. On diets containing casein as the protein source and sucrose as the carbohydrate, vitamin B_{12} has very little effect in protecting against growth retardation caused by thyroid feeding (Ershoff, '48, '49a, b). Betheil and Lardy ('49) did show a partial antithyrotoxic effect of vitamin B_{12} on a casein-sucrose diet but obtained a complete reversal of the growth inhibition caused by a 0.25% desiccated thyroid level in the ration only when a 10% level of whole liver powder was used. In a later communication Lewis, Tappan, Register, and Elvehjem ('50) reported that vitamin B_{12} only partially reversed the growth inhibition of thyroid in rats when a casein-sucrose ration was fed, but that this vitamin did produce significant growth responses when dex-

[1] Contribution 302 from the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles. This experiment was made possible by a research grant from The Best Foods, Inc., Bayonne, New Jersey.

[2] Present address: Department of Pharmacology, Medical School, Hospitals Centre, Birmingham, England.

trin, cornstarch, or defatted corn meal was used as the carbohydrate source.

Several excellent reviews of the literature relevant to the increased requirement for a number of known nutrients in the hyperthyroid animal have been made (Drill, '43; Betheil and collaborators, '47; Ershoff, '49b). In the latter report a comprehensive review was also given of material concerning the beneficial effects of fat in the diet of thyroid-fed animals.

It was shown by Ershoff ('49b) that the growth-inhibiting effect of thyroid on the rat fed a diet low in fat could be counteracted completely by the addition of fat to the diet when soybean meal was used as the protein component, and to only a limited extent when casein was used as a protein source. In an earlier report from this laboratory (Greenberg and Deuel, '50) it was shown that on a fat-free, low-thyroid diet, but one which inhibited the growth of the rat to a significant extent, the growth-inhibiting effect of the thyroid could be completely counteracted by a high level of fat.

The following experiment was designed to determine the effects of vitamin B_{12} on the growth of rats fed a fat-free diet without and with thyroid, as well as to determine the growth-augmenting effect of linoleic acid and of fat when low-fat thyroid diets containing casein as the protein source are fed.

EXPERIMENTAL

Weanling male rats of the Long-Evans strain,[3] aged 21 to 25 days, were housed for experimental purposes in individual metal cages with raised screen bottoms to minimize access to the feces. The rats were divided into 10 groups of 7 to 8 animals each. The basal diet employed in the study had the following percentage composition: casein,[4] 20; sucrose, 72; salt mixture, [5] 4; celluflour,[6] 4; and contained the

[3] Obtained from the Pacific Farms, Gardena, California.
[4] Vitamin-test, General Biochemicals, Inc., Chagrin Falls, Ohio.
[5] United States Pharmacopoeia XIV.
[6] Chicago Dietetic Supply House, Chicago, Illinois.

following micronutrients,[7] in milligrams per kilogram of diet: thiamine chloride hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 8; calcium pantothenate, 80; niacin, 10; 2-methyl-1,4-naphthoquinone, 2; folic acid, 4; biotin, 0.4; para-aminobenzoic acid, 2,000; inositol, 4,000; and choline chloride, 4,000. The fat-soluble vitamins were given orally twice weekly, made up in dosages of 0.1 ml of propylene glycol. The dosage per 0.1 ml was as follows: vitamin A alcohol, 333 U.S.P. units; calciferol, 32 U.S.P. units; and α-tocopherol, 2.8 mg.

The groups receiving the basal diets to which crystalline vitamin B_{12}[8] or desiccated thyroid powder,[9] or both, were added are indicated in table 1. Food and water were supplied ad libitum. However, food consumptions were recorded and these data are also presented in table 1, with determinations of the efficiency of the food eaten. The fat supplements were calculated so they could be administered 4 times weekly.

At the end of a 7-week growth period, representative groups of the rats fed the experimental diets were sacrificed and the weights of the thyroids, adrenals, testes, kidneys, and heart ventricles were determined.

RESULTS AND DISCUSSION

Vitamin B_{12} appeared to have no effect on the growth rates of rats being depleted of fat when added to the fat-free, sucrose-casein diet (groups I and II, table 1). Nor was there any significant difference when vitamin B_{12} was added at a level of 40 μg per kilogram of diet to a fat-free diet containing 0.05% of desiccated thyroid powder (groups III and IV, table 1). There was a noticeable decrease in the efficiency of the diet when thyroid was added. This decrease was not corrected to any great extent when vitamin B_{12} was added. Without thyroid in the diet, the efficiency of the basal diet

[7] The biotin was kindly furnished by Hoffmann-La Roche; the folic acid by Lederle Laboratories; the inositol by Corn Products, Inc.; and all other micronutrients, including the α-tocopherol, were supplied by Merck and Company, Inc.

[8] See footnote 7.

[9] Armour, U.S.P.

TABLE 1

The effect of crystalline vitamin B_{12}, when added at a level of 40 μg/kg of diet, of cottonseed oil,[1] and of methyl linoleate[2] on rats fed a fat-free diet containing 0.05% desiccated thyroid powder

GROUP NO.	SUPPLEMENT				NUMBER OF RATS	INITIAL BODY WEIGHT	GAIN IN BODY WEIGHT [4]		TOTAL FOOD INTAKE	EFFICIENCY OF DIET [5]
	Vitamin B_{12}	Thyroid [3]	Methyl linoleate	Cottonseed oil			4 weeks	7 weeks		
	μg/kg		mg/day	mg/day		gm	gm	gm	gm	
I	0	—	0	0	7	60.6	124.0	158.1 ± 8.2	708	5.92
II	40	—	0	0	8	54.7	112.3	156.1 ± 6.6	666	6.20
III	0	+	0	0	8	48.8	78.5	103.7 ± 4.9	628	4.37
IV	40	+	0	0	8	45.5	93.4 (7)	118.7 (7) ± 9.6	693	4.54
V	40	—	50	0	7	37.7	120.4	176.9 ± 6.2	658	7.06
VI	40	+	50	0	8	46.7	103.8	165.8 (6) ± 5.0	770	5.65
VII	40	—	0	100	7	42.4	128.6	183.0 ± 10.4	682	6.99
VIII	40	+	0	100	8	45.5	110.5	172.2 ± 6.7	750	6.31
IX	40	—	0	500	7 [6]	40.9	128.0 (6)	191.0 (5) ± 7.1	656	7.07
X	40	+	0	500	8	48.0	120.7	189.3 ± 8.0	778	5.98

[1] Wesson.

[2] Hormel Institute, Austin, Minnesota.

[3] Diet contained 0.05% of desiccated thyroid powder.

[4] Figures in parentheses indicate number alive at time indicated. Weight gains at 7 weeks also include the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

[5] Grams gain in weight per calories consumed × 100. The efficiency calculations include consideration of the fats fed as supplements.

[6] Of 7 animals starting group IX, one died within the first week and a second rat was accidentally killed during the 6th week.

was 5.92 when vitamin B_{12} was also absent and 6.20 when this vitamin was added. When the diet contained 0.05% of thyroid powder, the efficiency of the diet without vitamin B_{12} was reduced to 4.37, and to 4.54 with vitamin B_{12} added at a level of 40 μg per kilogram of ration.

The failure of vitamin B_{12} to show antithyrotoxic effects as measured by its effect in counteracting the growth-retarding action of thyroid, or by its effect on the efficiency of the diet, is in confirmation of similar results reported by Ershoff ('48, '49a, b) and by Lewis et al. ('50).

The growth-depressing effects of the thyroid are counteracted either by linoleate (group VI, table 1) or by whole fat (groups VIII and X, table 1). The fat-tested, refined cottonseed oil is reported in the literature, and has been determined by biological assay (Deuel, Greenberg, Anisfeld, and Melnick, '51), to contain about 50% of essential fatty acids.

The growth-depressing effects of the thyroid (group IV as compared to group II, table 1) are highly significant ($P<0.01$) and the counteraction of this effect by linoleate and by fats (groups VI, VIII, and X as compared to group IV, table 1) is also highly significant ($P<0.01$). At the highest level of fat used (500 mg/day) a complete counteraction of growth retardation by the addition of fat to a fat-free diet containing desiccated thyroid powder was demonstrated (equal growth in groups IX and X, table 1). These results are in confirmation of the earlier report of Greenberg and Deuel ('50). That the counteracting effect can be attributed to lowered food consumption with concomitant lowered thyroid intake in the groups receiving fat (Lewis et al., '50) has been refuted by the results obtained in this experiment and reported in table 1. In fact, the groups receiving the basal diet containing vitamin B_{12} and thyroid, supplemented with fat (groups VI, VIII, and X) consumed more thyroid than did the control group, IV. The fat supplement permits a greater utilization of the food when thyroid is present than is otherwise obtained by the rat; however, the efficiency of the diet was not as great in any of the thyroid-fed groups receiving

fats or the fatty acid as in the parallel groups not fed thyroid (table 1).

The average weights of ventricles, kidneys, testes, adrenals, thymus, and thyroid for control groups and for fat-fed groups with and without thyroid are presented in table 2. The ratios of organ weights to body weights were determined for each organ excised and the average results, with a statistical analysis, are likewise recorded in table 2.

Desiccated thyroid powder, even at the low level of 0.05% in the diet, causes a significant increase in heart, kidneys, and adrenal size, relative to body size, when the thyroid is added to a fat-free diet. The levels of fat fed (50 mg of methyl linoleate per day and 500 mg of cottonseed oil per day) do not counteract the enlargement of the heart but do reduce the relative size of the kidney. The reduction of kidney size ($P<0.01$) in relation to body size for animals fed thyroid in a fat-free diet becomes significant at the higher levels of fat supplementation (group X compared to group IV). Whether complete counteraction of the effect of thyroid on the heart and kidney could be accomplished by the inclusion of larger amounts of fat in a diet containing such a low level of thyroid has not been ascertained by this experiment. There was no significant effect of either vitamin B_{12} or of the low level of thyroid fed on the ratios of testes or thymus weight to body weight. However, the thymus ratio to body weight was increased somewhat in the presence of fat in the diet.

There is considerable similarity between the protective effect that fat had for immature rats fed thyroid under the conditions of this experiment and the effects that Ershoff ('47) and Betheil et al. ('47) have obtained with high levels of liver in the diet. Ershoff ('49b) was also able to show that fats had beneficial effects on rats fed higher levels of thyroid in the diet. It would seem probable that, since Ershoff was able to obtain significantly more growth by the feeding of liver than could be gotten by the inclusion of high levels of fat in the diet, a factor is present in liver which exerts protective effects for the immature rat fed thyroid.

TABLE 2

Effect of fat supplementation on organ weights of rats fed desiccated thyroid (0.05%) in a fat-free diet. (The ratio of organ weight (mg) to body weight (gm) is presented in parentheses under the organ weight average for each group)

GROUP NO.	BODY WEIGHT AT 7 WEEKS [1]	ORGAN WEIGHT WITH RATIO TO BODY WEIGHT AND STANDARD ERROR OF THE MEAN [2] GIVEN IN PARENTHESES					
		Ventricle	Kidneys	Testes	Adrenals	Thymus	Thyroid
	gm	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
II	211 (8)	778 (3.70 ± 0.13)	1808 (8.56 ± 0.23)	2537 (12.03 ± 0.25)	32 (0.154 ± 0.008)	231 (1.21 ± 0.01)	...
IV	165 (7)	923 (5.68 ± 0.35)	2319 (14.14 ± 0.51)	2133 (12.97 ± 0.48)	33 (0.207 ± 0.024)	191 (1.16 ± 0.17)	10.3 (0.066 ± 0.005)
V	215 (7)	818 (3.83 ± 0.17)	1948 (9.07 ± 0.49)	2475 (11.62 ± 0.37)	36 (0.171 ± 0.008)	313 (1.49 ± 0.16)	...
VI	214 (6)	1187 (5.55 ± 0.12)	2877 (13.40 ± 0.61)	2647 (12.32 ± 0.26)	38 (0.174 ± 0.010)	248 (1.15 ± 0.11)	10.3 (0.048 ± 0.001)
IX	232 (5)	800 (3.45 ± 0.13)	1905 (8.28 ± 0.35)	2559 (10.99 ± 0.44)	40 (0.175 ± 0.013)	417 (1.82 ± 0.39)	...
X	238 (8)	1298 (5.49 ± 0.38)	2748 (11.58 ± 0.29)	2713 (11.41 ± 0.27)	36 (0.150 ± 0.013)	366 (1.52 ± 0.13)	12.6 (0.053 ± 0.003)

[1] Number of animals studied given in parentheses.

[2] Standard error of the mean calculated by formula given in footnote 4, table 1.

The beneficial effects of fat may be attributable to its stimulating effect upon the intestinal flora, causing it to synthesize a factor or factors similar to that found in liver. The quantitative differences between the effects of fat as a protective agent found by Ershoff and those observed in the experiment reported here are probably due to differences in the levels of thyroid fed and, consequently, to amounts of the factor or factors required by the animal. It is of particular interest at this point to note that the protective factor found in liver is not water-soluble and is only partially soluble in fat solvents (Ershoff, '48; Betheil and Lardy, '49).

In view of the results reported by Kunkel and Williams ('51) to the effect that fat and essential fatty acids have direct effects on enzymatic activities in body tissues, one cannot rule out the possible direct effect of the essential fatty acids, of fat itself, or of some unknown factor in fat which in turn may be concentrated or bound in liver.

SUMMARY

Crystalline vitamin B_{12} has no appreciable effect in counteracting the growth-retarding action in rats of desiccated thyroid powder in a fat-free, sucrose-casein diet.

Methyl linoleate and cottonseed oil administration protect the immature rat against growth retardation when the rat is fed a fat-free, sucrose-casein diet to which thyroid (0.05% of the diet) has been added.

The levels of essential fatty acid (50 mg of linoleate per day) and cottonseed oil (500 mg per day) which were fed to hyperthyroid rats do not protect the animal against enlargement of the heart and protect it only partially against enlargement of the kidney.

The possible relationship between the protective activity of fat and that of liver for immature rats fed thyroid is discussed.

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HEPATIC NECROSIS INDUCED BY DIETARY MEANS

VI. THE EFFECT OF VARYING THE LEVEL AND NATURE OF PROTEIN AND THE LEVEL OF FAT ON THE DEVELOPMENT OF LIVER NECROSIS [1]

JOHN R. McLEAN AND J. M. R. BEVERIDGE
Department of Biochemistry, Queen's University, Kingston, Ontario, Canada

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In 1935 Weichselbaum reported that rats fed a diet deficient in cystine and methionine became ill and died in about 6 weeks. On post mortem examination the livers of these animals "showed definite haemorrhages throughout the liver." Although no histological examinations of these livers were made, the lesion described was probably that under investigation here; namely, acute massive liver necrosis. The first clear description of liver necrosis was given in 1939 by Gyorgy and Goldblatt, who fed to rats a diet designed to produce cirrhosis of the liver and found that some of the animals died of acute necrosis of that organ. The distinction between the etiology of liver cirrhosis and acute massive liver necrosis was made a few years later by Daft, Sebrell and Lillie ('42). They found that methionine or choline prevented fatty infiltration and cirrhosis, whereas cystine exerted no prophylactic action and indeed it actually increased the severity of the fatty infiltration and resulting fibrosis. Liver necrosis, on the other hand, was prevented by cystine and methionine but choline exerted no prophylactic action.

[1] The work reported in this paper was submitted in partial fulfillment of the requirements for the degree of Master of Arts by John R. McLean. A preliminary report of this investigation was given before the Canadian Physiological Society at Kingston, Ontario, October 26, 1951.

In experiments of a similar nature, Hock and Fink ('43a, b) utilized various yeasts to supply the protein moiety of the diet and found that the incidence and severity of hepatic necrosis increased as the protein sulfur in the diet diminished. Following these reports many workers (Irving and Schwarz, '46; Neuberger and Webster, '47; Sellers, Lucas and Best, '48; Rao, '48; Hall and Drill, '49) attempted to produce liver necrosis in rats using diets low in protein and deficient in the sulfur-containing amino acids, but their efforts were marked by a high degree of inconsistency. The problem was clarified further in 1949 by Himsworth and Lindan and by Gyorgy and Goldblatt, who conclusively demonstrated that diets designed to induce hepatic necrosis must also be deficient in alpha-tocopherol. This point was also noted by Abell and Beveridge in the same year. The latter workers ('49, '51) also reported that the inclusion of cod liver oil in the basal diet increased its necrogenicity, a circumstance presumably dependent upon its antagonistic action on alpha-tocopherol in the animal body (Mackenzie et al., '41).

Utilizing alkali-treated casein as the source of dietary protein, Schwarz produced a type of liver necrosis of different etiology, which he demonstrated could be prevented by alpha-tocopherol or xanthine ('44a, b) and he also showed that high levels of cod liver oil (10 and 20%) accelerated the production of the lesion ('48).

Other dietary factors have been shown to influence the development of liver necrosis. Gyorgy, Stokes, Smith and Goldblatt ('50) have reported that aureomycin delays the development of liver necrosis, a finding that has been confirmed by Abell and Beveridge ('51), who also demonstrated that sulphaguanidine has a similar action. The latter workers found also that synthetic vitamin K decreased the necrogenicity of a diet designed to induce necrosis. Handler and Follis ('48) have reported that addition of thyroid to a diet greatly accelerated its capacity to produce hepatic damage, whereas thiouracil had an opposite effect. Similar results were obtained by our group (Abell and Beveridge, '51) using propyl-

thiouracil in place of thiouracil. Further work by McLean and Beveridge ('52) led to the conclusion that the level of thyroid activity is a subsidiary, not a determining, factor in the production of this lesion.

Himsworth and Glynn ('44), utilizing diets high in fat or in carbohydrate, concluded that these dietary modifications had no effect on the development of necrosis, but that the absolute protein intake was the prime consideration in determining the necrogenicity of a diet. In a review of the literature relating to liver lesions due to dietary imbalance, Gyorgy ('49) indicated that dietary fat had no effect or was injurious, although in an earlier paper the same worker noted that there was a tendency for a greater incidence of necrosis to occur when the fat component of the diet was highly unsaturated. However, this point was not definitely established. Goettsch ('51) has recently concluded that varying the lard content of a diet from 0 to 20% had no effect on its necrogenicity. The diet containing no lard was not fat-free, a point that should be emphasized in view of the results obtained in our investigation.

However, insofar as the authors are aware, there has not been reported any extensive investigation into the effects of varying the dietary levels of fat, protein and hence necessarily carbohydrate, on the production of liver necrosis.

It was felt that further information regarding the effects of varying the amounts of these three important dietary components would further elucidate the etiology of the lesion and facilitate subsequent experimental work. Accordingly, the following experiments were performed to obtain additional information on these points.

EXPERIMENTAL PROCEDURE

One hundred and five male rats of the Sprague-Dawley strain were divided into groups of 5 of an average initial weight of 54.7 (S.D. = 3.7) gm. These were housed in individual cages with mesh screen floors and were offered daily approximately 8 gm of the test diets. The composition

of the basal diet is given in table 1. It may be noted that all the vitamins readily available in crystalline form were incorporated into the sugar vitamin mixture, despite the fact that under normal circumstances it is well known that the rat does not require certain of these compounds in the diet. This was done in order to minimize the possibility that the feeding of the basal diet, which was known to be deficient

TABLE 1

Composition of basal diet

CONSTITUENTS	AMOUNT
	%
Brewers' yeast (U.S.P., Nutritional Biochemicals Co.)	18
Lard	5
Sucrose	70.98
Salts	3
Sugar vitamin mixture[1]	1
Cellu flour	2
Cod liver oil concentrate (Ayerst, McKenna and Harrison; 50,000 I.U. vitamin D and 200,000 I.U. vitamin A per gram)	0.02

[1] The sugar vitamin mixture had the following percentage composition (the figures shown in brackets are the amounts contained in 8 gm of diet): Thiamine hydrochloride 0.025 (0.02 mg), riboflavin 0.03125 (0.025 mg), pyridoxine 0.025 (0.02 mg), calcium pantothenate 0.125 (0.1 mg), nicotinic acid 0.1 (0.08 mg), folic acid 0.005 (0.004 mg), B_{12} 0.00006 (0.48 μg), ascorbic acid 6.25 (5 mg), inositol 10 (8 mg), biotin 0.0375 (0.03 mg), para-aminobenzoic acid 0.1 (0.08 mg), menadione 0.08 (0.064 mg), choline 30 (24 mg), icing sugar 53.22.

in protein, would lead to a conditioned deficiency. It is well known, for example, that a deficiency of tryptophan increases the need for niacin in the diet (Krehl et al., '46). Furthermore Roberts and Spiegl ('47) have shown that protein, and specifically methionine and cystine, increased ascorbic acid synthesis by the rat. Presumably a diet deficient in these amino acids, such as that utilized in the present work, might lead to a significant decrease in ascorbic acid synthesis.

The fat content was varied from 0 to 70%, the groups receiving these diets being designated as F0, F5, F10, F20, F40, and F70. The diet given to group F0 was intended to be free from fat, and in order to achieve this purpose the yeast component was extracted three times with 5 volumes of hot 95% alcohol.

It has been our experience and also the experience of other workers that liver necrosis always occurred more readily on diets in which the protein component was supplied in the form of yeast rather than in the form of casein. For this reason the protein moiety was supplied in the form of yeast or of casein, the yeast being varied in amount from 0 to 60% (Y0, Y5, Y10, Y20, Y30, Y40, Y60) and the casein from 0 to 7% (C0, C1, C2, C3, C4, C5, C6, C7). All modifications in the basal diet were made at the expense of carbohydrate. The experiment was terminated after an experimental period of 172 days. Although the presence of massive liver necrosis could usually be detected readily in the gross, the final diagnosis was based always on a microscopic examination of sections, stained with hematoxylin and eosin, from the three main lobes of the liver.

RESULTS AND DISCUSSION

The incidence of liver necrosis and the time required for its development on the various diets are shown in tables 2 through 4.

The modification of the protein moiety of the basal diet in the form of yeast (see table 2) revealed that when there was no protein in the ration the animals survived an average period of only 35 days and died of causes other than liver necrosis. The addition of 5 and 10% yeast caused the death of all animals due to hepatic necrosis at average times of 64 and 73 days, respectively. Additional information on the effect of varying levels of yeast may be obtained from an examination of data relating to group F5 (see table 4) fed a diet which contained 18% yeast, a ration which corresponds to the basal diet. Again the incidence of necrosis

was 100% and survival time was an average of 73 days. In group Y20, the incidence of necrosis was only 40% and the average time at which the lesion caused death was 90 days. None of the rats receiving more than 20% yeast in the diet died of hepatic damage. Apparently a level of 18 to 20% yeast, of the type used here, is a crucial one, above which the necrogenicity of the diet is markedly reduced. The results would appear to militate against acceptance of the thesis that there is a toxic factor in yeast (Gyorgy, Rose, Tomarelli and Goldblatt, '50) and to support the belief that there are present protective factors only.

TABLE 2

The effect of yeast on the development of liver necrosis

GROUP	RATS DYING OF NECROSIS OR KILLED IN EXTREMIS			RATS DYING OF OTHER CAUSES			TOTAL INCIDENCE OF NECROSIS	
	Number of rats	Days on diet		Number of rats	Days on diet		Number	%
		Ave.	Range		Ave.	Range		
Y0	0	..	..	5	35	(19–49)	0/5	0
Y5	4	64	(57–71)	1	33	..	4/5	80
Y10	5	73	(57–86)	0	..	..	5/5	100
Y20	2	90	(64–117)	0	..	..	2/5	40
Y30	0	..	..	0	..	..	0/5	0
Y40	0	..	..	1	86	..	0/5	0
Y60	0	..	..	2	10	(5–11)	0/5	0

When varying amounts of casein were utilized as the protein component of the diet, the incidence of liver necrosis remained low or at zero throughout (see table 3). Only at levels of 3 and 4% of casein did any hepatic damage occur, and even in these cases only one of the group was affected. The low incidence of liver necrosis observed when casein served as the protein moiety of the diet is in line with our previous experience and also with that of Gyorgy and Goldblatt ('49). It must be realized, however, that the necrogenicity of a diet depends upon a number of factors besides the sulfur-containing amino acids and alpha-tocopherol, one of the more important governing factors being the level

of cod liver oil in the diet (Abell and Beveridge, '49, '51). Since vitamins A and D were supplied in the form of a cod liver oil concentrate at a level of only 0.02%, it is readily understandable why the necrogenicity of these diets was so low. Other workers utilizing rations containing 5% casein, and indeed supplemented with small amounts of alpha-tocopherol, have reported moderately high incidences of necrosis (Handler and Follis, '48). It is very likely that these results were due to the incorporation of a relatively high level of cod liver oil in the diet (4%). More recently Goettsch ('51),

TABLE 3

The effect of casein on the development of liver necrosis

GROUP	RATS DYING OF NECROSIS OR KILLED IN EXTREMIS			RATS DYING OF OTHER CAUSES			TOTAL INCIDENCE OF NECROSIS	
	Number of rats	Days on diet		Number of rats	Days on diet		Number	%
		Ave.	Range		Ave.	Range		
C0	0	..		5	35	(19–49)	0/5	0
C1	0	..		5	53	(45–66)	0/5	0
C2	0	..		5	45	(14–99)	0/5	0
C3	1	136		3	94	(78–122)	1/4 [1]	25
C4	1	85		1	71	..	2/5 [2]	40
C5	0	..		0	..	..	0/5	0
C6	0	..		0	..	..	0/5	0
C7	0	..		0	..	..	0/5	0

[1] Data on one animal lost.

[2] One animal sacrificed at 172 days showed liver necrosis.

using animals depleted of alpha-tocopherol, reported that liver necrosis occurred with a dietary casein level of as high as 11.6%. In addition to the factor of tocopherol depletion, cod liver oil was incorporated in the ration at a level of 2%.

It is of interest to note that despite the fact that a level of 18% yeast (see group F5, table 4) supplied approximately 130 mg of methionine and 94 mg of cystine per 100 gm of diet, making a total of 2.5 millimoles of amino acid sulfur, the incidence of necrosis was 100%, whereas with a diet containing 5% casein (see group C5, table 3) which supplied 146 mg methionine and 24 mg cystine per 100 gm diet, making a

total of 1.2 millimoles of amino acid sulfur, the incidence of necrosis was zero. It would therefore appear that the difference in necrogenicity of diets containing yeast or casein cannot be attributed to their differing concentrations of the sulfur-containing amino acids.

Since both yeast (Gyorgy, Rose, Tomarelli and Goldblatt, '50) and casein (Mason and Bryan, '38) have been shown to be essentially devoid of alpha-tocopherol, it would appear that a factor or factors other than those mentioned are involved in the etiology of acute massive liver necrosis. Further evidence for this statement may be found in a paper

TABLE 4

The effect of fat on the development of liver necrosis

GROUP	RATS DYING OF NECROSIS OR KILLED IN EXTREMIS			RATS DYING OF OTHER CAUSES			TOTAL INCIDENCE OF NECROSIS	
	Number of rats	Days on diet		Number of rats	Days on diet		Number	%
		Ave.	Range		Ave.	Range		
F0	0	..	..	0	..	..	0/5	0
F5	5	73	(38–157)	0	..	..	5/5	100
F10	5	59	(42–71)	0	..	..	5/5	100
F20	5	42	(25–53)	0	..	..	5/5	100
F40	5	38	(25–54)	0	..	..	5/5	100
F70	5	32	(19–42)	0	..	..	5/5	100

by Lindan and Work ('51), who analyzed two yeasts of widely varying necrogenicity and found that their methionine and cystine contents did not differ significantly. The analyses made by these workers did not permit them to arrive at any definite conclusion with regard to the reason for the differences in necrogenicity of the two yeasts.

The results obtained in this experiment with the use of varying amounts of casein and yeast might be explained on a basis similar to that proposed by Griffith and Mulford ('41) in their study of conditions affecting the accumulation of fat in the liver due to a dietary deficiency of lipotropic factors. These workers advanced the theory that the better a diet is from a nutritional standpoint — apart from lipo-

tropic factors — the greater is the demand for labile methyl groups, thus leading to a greater deposition of fat in the liver. It is possible that an analogous explanation may be valid for the phase of metabolism under study in this paper. Such a thesis would explain why no necrosis developed in animals receiving a diet devoid of protein. With regard to casein, there was no necrosis until levels of 3 and 4% were reached, and beyond this concentration necrosis again failed to appear. Presumably at low levels of protein the general level of metabolic activity is so low that an insufficient amount of the toxin responsible for the acute liver injury is produced, or a sufficient amount of the substances responsible for the detoxification process is available or becomes available due to catabolism of body tissue. At higher levels of protein, the general level of metabolic activity is raised and, due to the increased demand for protective factors, such as methionine, cystine and alpha-tocopherol, relatively less of these substances are available to preserve the liver in a normal state, hence fatal liver damage occurs. With a further increase in protein, some of these protective factors are present at a sufficiently high level to overcome the effects of the toxin responsible for the hepatic necrosis.

The incidence of necrosis on the diets containing variable amounts of fat is shown in table 4. All animals fed rations containing from 5 to 70% fat died of liver necrosis, but the time required for the development of the lesion increased with decreasing fat content, a fact which was supported by a statistical analysis of the results. The "P" values obtained from Fisher's table of "t" are as follows: F5 versus F10, $P = 0.40$; F5 versus F20, $P = 0.188$; F5 versus F70, $P = 0.018$; F10 versus F20, $P = 0.048$; F10 versus F40, $P = 0.03$; F10 versus F70, $P = 0.01$. The foregoing data indicate that under the experimental conditions used no difference could be demonstrated between the effects of feeding rations F5 and F10, but that, with the exception of F5 versus F20, the necrogenicity of these diets was significantly different from that of all other diets containing higher levels of fat. The

successive differences in necrogenicity noted between diets F20, F40 and F70 could not be shown to be statistically significant.

All the animals fed the diet free from fat survived the experimental period, apparently in good health, although they did show some of the signs of essential fatty acid deficiency. Gross and microscopic examinations of the liver of these animals revealed no evidence of liver necrosis. Under the conditions of this experiment, the presence of some factor in the fat moiety of the diet appears to be required for the production of liver necrosis. There are a number of possible explanations for the foregoing result. For example, the work of Gottlieb, Quackenbush and Steenbock ('43) demonstrated that the requirement of the rat for alpha-tocopherol is decreased as the fat content of the diet is lowered. It follows that the results obtained in the present investigation might be explained on the basis of an increased demand for alpha-tocopherol due to an increase in dietary fat. On the other hand, as Mason and Filer have shown ('47), signs of vitamin E deficiency can be produced on diets devoid of fat.

A paper by Dam and Granados ('51), published recently, describes results similar to those reported here on the effect of diets free from fat in preventing the appearance of hepatic necrosis. The absence of hepatic damage extended over an experimental period of 70 days, which may be contrasted with the experimental period in the present report of 172 days. It should be emphasized that a number of dietary modifications can delay the appearance of liver necrosis and a period of 70 days is a remarkably short time on which to reach the conclusion that the absence of fat prevented the development of hepatic damage. This is particularly true since the diet containing fat caused only a 50% incidence of necrosis, as reported in their work. Indeed we still entertain the possibility that had our experiments been prolonged beyond 172 days, acute massive hepatic necrosis might have developed in the animals on the diet free from fat.

A remote possibility that might also help to explain the necrogenic effect of fat would be the development of rancidity in the diets containing this component. It has long been known that rancid fats effect a destruction of alpha-tocopherol (Evans and Burr, '27; Mattill, '27), and indeed this factor has already been shown to play a role in potentiating the necrogenic action of a diet (Abell and Beveridge, '49). Apart from the fact that the diets were made up at short intervals and stored in tightly closed containers at approximately 4°C. in order to minimize the development of rancidity, we would like to discount this possible explanation for the present results on the basis of the following data obtained in previous work. When the anti-oxidant, hydroquinone, was incorporated into a basal necrogenic diet at a level of 0.1% along with 0.5% each of the emulsifying agents, span 80 and tween 80, which were included in order to allow the anti-oxidant to enter the fat phase, there was no effect on the necrogenicity of the diet. The incidence of necrosis was 10/10 for both the basal and supplemented diets and the average time at which death occurred due to liver necrosis was 49 and 51 days, respectively.

The argument might be made that the absence of necrosis in the group fed the diet free from fat was not due to the absence of this dietary component but rather to the elimination of a necrogenic factor from the yeast by means of the hot alcohol extraction. However, in view of the decreasing necrogenicity of diets containing decreasing levels of fat, it seems unlikely that the alcohol extraction of the yeast played any major role in the prevention of liver necrosis apart from the removal of lipids from the yeast. Experiments are now being carried out to amplify this point.

It has been our experience (McLean and Beveridge, '52) that rats on a necrogenic diet consistently show a blanching of the incisors, a sign that has been described as being indicative of a deficiency of alpha-tocopherol (Davies and Moore, '41). The incisors of the rats maintained on the diet devoid of fat retained their original orange-brown pigmenta-

tion. Both cod liver oil and lard have been reported to accentuate the dental depigmentation seen in vitamin E deficiency, and in the case of cod liver oil it has been shown that the highly unsaturated fatty acid fraction is responsible for its action (Dam and Granados, '45), a fact which suggests that this fraction might also be responsible for the necrogenicity of the fat moiety of the diet.

It is axiomatic that when one component of a diet is changed, at least one other must also be varied, and under such circumstances it is sometimes difficult to allocate responsibility for the effects obtained. In the present investigation dietary alterations were made with the prime purpose of determining the effect of differing levels of protein and fat. In both cases the changes were accompanied by simultaneous inverse modifications in the level of carbohydrate. This fact permits an assessment of the effect of altering the latter dietary component. In the yeast series going from Y5 to Y60 the carbohydrate of the ration becomes reduced and the necrogenicity of the diet is correspondingly decreased. On the other hand, in the fat series going from F0 to F70, although the carbohydrate level is similarly affected, the necrogenicity of the ration is increased. These results indicate that sucrose is relatively inert in the process under investigation.

SUMMARY

1. The protein, fat, and carbohydrate components of a basal necrogenic diet have been fed at various levels to groups of 5 male weanling rats of the Sprague-Dawley strain.

2. The protein moiety was varied by feeding yeast at levels of from 0 to 60% and casein at levels of from 0 to 7%. No liver necrosis occurred in groups fed rations devoid of protein or containing casein at levels of 1 or 2%. At concentrations of 3 and 4% casein there was a low incidence of liver damage, and none at levels of greater than 4%. When yeast was fed at concentrations of 30% or above, no liver damage was observed. Diets containing 5 to 18% yeast caused the death of all animals, due to liver necrosis. A comparison of the

foregoing results indicates that some factor other than those previously described — cystine, methionine and alpha-tocopherol — has a profound effect on the development of acute massive hepatic necrosis.

3. Increasing levels of fat produced concomitant increases in the necrogenicity of the basal diet. A ration free from fat prevented the development of liver necrosis.

4. Variation in the carbohydrate level had no discernible effect on the necrogenicity of the basal diet.

ACKNOWLEDGMENTS

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Erratum note to be cut out and pasted on page 55 of the May 1952 issue (article by McLean and Beveridge: HEPATIC NECROSIS INDUCED BY DIETARY MEANS VI).

Editorial Note

ADDENDUM

Re type of yeast: Information has been received indicating that this yeast was incorrectly labeled by the Nutritional Biochemicals Corporation and should have been termed "Primary Dried Yeast" (Saccharomyces cerevisiae).

SOME RELATIONSHIPS AMONG DIETARY STEROLS, PLASMA AND LIVER CHOLESTEROL LEVELS, AND ATHEROSCLEROSIS IN CHICKS

D. W. PETERSON, C. W. NICHOLS, JR. AND E. A. SHNEOUR
WITH THE TECHNICAL ASSISTANCE OF H. GAFFEY, R. ROBBINS AND N. PEEK
Division of Poultry Husbandry, College of Agriculture, and Division of Physiology, School of Medicine, University of California, Berkeley

ONE FIGURE

(Received for publication February 4, 1952)

Plant sterols are usually considered to be inert dietary constituents, since investigations with several animals have shown that there is little, if any, absorption of these sterols from the gastrointestinal tract. Thus, little, if any, absorption of ergosterol could be demonstrated in rats, mice, rabbits and dogs. No absorption of sitosterols could be detected in rabbits and dogs, and no increase in liver sterols was found after feeding stigmasterol and brassicasterol to mice (Schoenheimer, '31; Behring and Schoenheimer, '30; Schoenheimer et al., '30). Sperry and Bergmann ('37) were unable to demonstrate an increase in liver sterols after feeding of sitosterols and stigmasterol to mice.

Cholesterol, on the other hand, is readily absorbed by these animals, as well as by the chicken, especially when they are supplied with extra fat in the diet. Prolonged feeding of high cholesterol diets to chicks results in elevated plasma cholesterol, deposition of large amounts of cholesterol in the liver, and atheromatosis (Dauber and Katz, '42; Stamler et al., '50).

Recently it has been shown that soybean sterols, fed to chicks together with cholesterol, prevent hypercholesteremia

and decrease markedly the deposition of cholesterol in the liver (Peterson, '51).

The present report describes the results of further experiments, involving the feeding of these diets over a prolonged period and a study of their effects on the development of atheromatosis in the aortas of the chicks.

METHODS

Diets

Three groups of 44 male S. C. White Leghorn chicks evenly distributed as to weight were placed on one of the three experimental diets at two weeks of age.

The diets were constituted as follows:

Diet 1: (basal) ground barley 25.5, ground corn 22, ground wheat 10, wheat bran 10, soybean oil meal 10, fish meal 7.5, dried whey 2.5, dried skim milk 2.5, liver meal 2, ground limestone 1.5, steamed bonemeal 1.2, sodium chloride 0.5, Mn $SO_4 \cdot H_2O$ 0.025, fortified fish oil (2,250 A-300 D per gram) 0.25, cotton seed oil [1] 4.5 gm per 100 gm and riboflavin 2 mg per kilogram of diet.

Diet 2 was identical with diet 1 except for the addition of 1% cholesterol replacing an equal amount of barley.

Diet 3 was also the same as diet 1, except for 1% added cholesterol and 1.3% mixed soy sterols,[2] replacing an equal amount of barley.

The sterols were dissolved in the cottonseed oil, heated with stirring until they just dissolved, then allowed to cool and the mixture incorporated into the diet.

Analytical

Heparinized blood samples were drawn from the alar vein from half of the chicks on each diet on alternate weeks beginning with the 4th week. Pooled samples of plasma from

[1] Wesson oil.

[2] The mixed soy sterols supplied by Distillation Products Industries contained a minimum of 95% sterols, principally sitosterols and stigmasterol.

three chicks were analyzed for total cholesterol by the method of Schoenheimer and Sperry as modified by Sperry and Webb ('50). Livers taken at autopsy were prepared as previously described by Peterson ('51) and analyzed by the same method.

Autopsy procedure

Approximately 10 chicks on each diet were sacrificed by cervical fracture at intervals of 10, 15, 24 and 28 weeks on the experiment. At autopsy, birds of the three experimental groups were mixed indiscriminately and examined as unknowns by a single observer who was unaware of the purpose of this investigation. The thoracic aortas were dissected down to the level of the adrenal glands. The abdominal segments of the aortas, including the iliac branches, were also dissected out. The vessels were opened longitudinally and the intima carefully inspected for atheromatous plaques. Lesions, if any, were graded zero to 4 according to the following criteria:

0: no gross changes or yellowish intimal discoloration;
0.5: extensive coalescing intimal discoloration, lacking a grossly elevated intimal surface;
1: definite plaques less than 3 mm^2, white to cream-colored, with raised intimal surface;
2: definite plaques greater than 3 mm^2, cream to light yellow in color;
3: numerous plaques greater than 3 mm^2, yellow in color;
4: severe gross atheromatosis (lesions yellow in color, raised and calcified on gross examination).

EXPERIMENTAL AND RESULTS

Cholesterol levels

No significant differences in rates of growth of the chicks were observed on the three diets. Mortality from all causes was three birds on diet 1, 6 on diet 2 and 6 on diet 3. Figure 1 shows average plasma total cholesterol values for pooled samples from the 4th to the 28th week on the diets. The

number of birds represented by each value is shown in table 1. Comparable curves for earlier periods on similar diets have been reported elsewhere (Peterson, '51). The broken and solid lines for each diet represent groups of birds sampled on odd and even weeks, respectively. Due to depletion in numbers by autopsy, all birds remaining after the 15th week were sampled at one time.

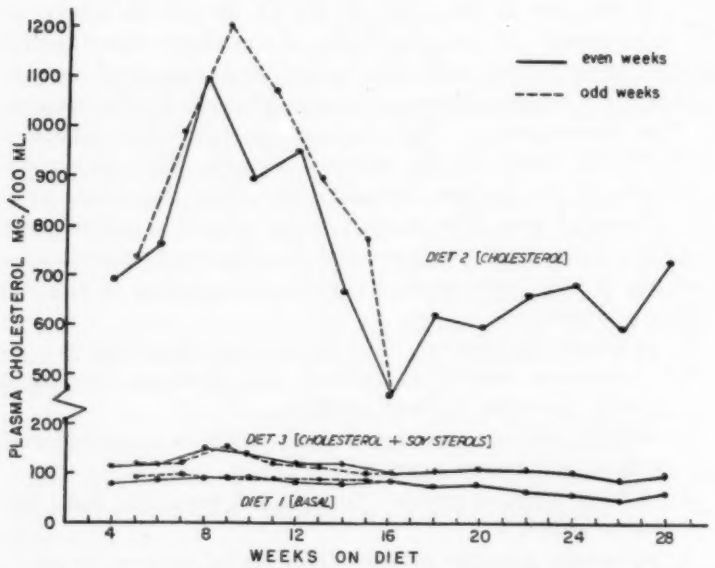

Fig. 1 Plasma total cholesterol levels on three experimental diets.

Chicks on diet 2 (1% cholesterol) showed maximum plasma cholesterol values between 8 and 10 weeks, with a fall thereafter until the 16th week, after which plasma cholesterol was maintained at a lower level than the previous maximum. These results are similar to those previously reported by Rodbard et al. ('51).

The birds fed diet 3 (1% cholesterol plus 1.3% soy sterols) showed consistently a slightly higher plasma cholesterol level than those fed diet 1 (control). However, the level was very

low compared with the values obtained for birds fed diet 2, which contained the same amount of cholesterol. This difference in cholesterol levels suggests that addition of the soybean sterols to the diet resulted in decreased absorption of dietary cholesterol.

Table 2 shows liver total cholesterol levels for birds sacrificed at 10, 15, 24 and 28 weeks on the diets. The values for the group of birds sacrificed at 10 weeks have been reported previously (Peterson, '51). They are included in the table for comparison. Livers of birds fed diet 2 (cholesterol) gave higher cholesterol values at 10 weeks on the dry weight basis than in the subsequent periods. This corresponds with the peak values for plasma cholesterol. The livers taken at 15,

TABLE 1

Number of birds included in each value in figure 1

WEEKS ON DIET	4	5	6	7	8	9	10	11	12	13	14	15	16	18	20	22	24	26	28
Diet 1	15	19	21	19	21	16	21	16	12	19	18	7	24	24	24	24	24	12	12
Diet 2	15	21	21	20	20	20	20	20	11	19	17	7	23	21	21	21	20	8	10
Diet 3	15	21	21	21	21	21	20	21	12	20	17	9	20	19	19	16	19	9	9

24 and 28 weeks contained somewhat less cholesterol, on a dry weight basis, than those at 10 weeks but averaged about 7 times the level of the controls. In contrast, the livers from birds fed both soy sterols and cholesterol had cholesterol values which averaged about twice those of the controls.

Observations on the vascular lesions

The data on the vascular lesions are summarized in table 3. Under the conditions of the experiment, the occurrence of spontaneous atherosclerosis in the control birds (diet 1) was very low. A high incidence of atherosclerosis was induced in birds fed diet 2 (1% cholesterol) and there was a progressive increase in the frequency, severity and extent of the lesions with increasing age. Judged without microscopic examination of the aortic lesions, and using the criteria for

gross grading given above, the extent and severity of the lesions in the thoracic and abdominal aortas were about equal. For the entire experimental period the average grade of gross atherosclerosis of the thoracic aortas for chicks on diet 2 was 1.5 and that of the abdominal aortas 1.7. Judgment

TABLE 2

Liver cholesterol levels

WEEKS ON DIET	10	15	24	28
	Mean total cholesterol as % wet wt. ± standard deviation [1]			
Diet 1 Basal	0.314 ± 0.043 (9)[2]	0.270 ± 0.046 (7)	0.338 ± 0.027 (11)	0.317 ± 0.036 (13)
Diet 2 1% cholesterol	2.61 ± 0.64 (9)	2.65 ± 0.44 (7)	2.38 ± 0.57 (9)	2.49 ± 0.51 (10)
Diet 3 1% cholesterol 1.3% soy sterols	0.510 ± 0.104 (8)	0.604 ± 0.107 (9)	0.615 ± 0.098 (10)	0.610 ± 0.112 (9)
	Mean total cholesterol as % dry wt. ± standard deviation			
Diet 1	0.969 ± 0.167 (9)	1.16 ± 0.15 (7)	1.15 ± 0.16 (11)	1.07 ± 0.10 (13)
Diet 2	9.48 ± 0.93 (9)	8.53 ± 1.35 (7)	7.40 ± 1.59 (9)	7.58 ± 1.44 (10)
Diet 3	1.62 ± 0.32 (8)	2.49 ± 0.43 (9)	2.07 ± 0.34 (10)	2.11 ± 0.40 (9)

[1] Standard deviation = $\sqrt{\dfrac{n\sum_{i=1}^{n} x_i^2 - \left(\sum_{i=1}^{n} x_i\right)^2}{n(n-1)}}$

[2] Figures in parentheses represent number of birds.

of the severity of the atherosclerosis on the basis of gross grading has been justified by Horlick and Katz ('49), who have pointed out that "gross inspection correlated closely with the microscopic appearance, and that gross grading was the more reliable method as it precluded the possibility of the section being inadvertently cut from an uninvolved portion of the aorta."

Although some atherosclerotic lesions were found in birds fed the diet containing mixed soy sterols and cholesterol, both the severity and the extent of the lesions occurring in this group were much less than those in birds fed 1% cholesterol alone (see table 3).

In treating these results statistically according to Fisher's exact method for 2×2 tables (Fisher, '34), the only criterion used was presence or absence of aortic lesions, without regard

TABLE 3

Effect of feeding cholesterol and soy sterols for varying periods on development of atherosclerosis

DIET	LENGTH OF TIME ON DIET	TOTAL NO. OF BIRDS EXAMINED	THORACIC AORTA			ABDOMINAL AORTA		
			No. of birds with lesions	Degree of athero-sclerosis	Aver-age grade	No. of birds with lesions	Degree of athero-sclerosis	Aver-age grade
	weeks							
I	10	7	0	0	0	0	0	0
	15	7	0	0	0	0	0	0
	24	11	0	0	0	0	0	0
	28	13	0	0	0	2	0–2	0.27
II	10	9	6	0–3	1.2	3	0–3	0.6
	15	7	7	1–3	1.9	5	0–3	1.6
	24	10	5	0–3	0.75	5	0–4	1.4
	28	10	8	0–4	2.1	8	0–4	2.3
III	10	7	0	0	0	0	0	0
	15	9	1	0–1	0.11	0	0	0
	24	10	1	0–2	0.2	3	0–3	0.55
	28	9	0	0	0	2	0–2	0.33

to their severity. On the assumption that the same probability of occurrence of at least one lesion in the aorta of an individual bird holds whether it is fed diet 2 or diet 3, the probability of a difference in the proportion of lesions as great as that actually observed between birds on these two diets is less than 0.0002 for each artery.

DISCUSSION

The results obtained by the inclusion of soybean sterols in the cholesterol-rich diet are not explained by the data

presented above, although these data suggest that the mechanism involved may be one of interference with cholesterol absorption. Previous experiments have shown that a similar effect may be obtained by the addition of a sitosterol fraction of soybean sterols to the cholesterol-rich diet (Peterson, '51). Sperry and Bergmann ('37) found that cholesterol levels were lower in the livers of mice fed sitosterols than in control mice, and suggested that sitosterols might interfere with reabsorption of cholesterol secreted into the intestinal tract.

The possibility must be considered that some unidentified constituent of the soy sterol mixtures is involved.

SUMMARY

The inclusion of soybean sterols in a high (1%) cholesterol diet prevented over a period of 28 weeks the usual hypercholesteremic response obtained with chicks on such a diet without added soybean sterols. The presence of soybean sterols in the diet also decreased the deposition of cholesterol in the livers of the chicks. There was a highly significant decrease in the incidence of atherosclerosis in chicks fed the soybean sterol-cholesterol diet.

The possibility is discussed that one or more of the constituents of the soybean sterols interferes with cholesterol absorption.

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COBALT METABOLISM OF YOUNG COLLEGE WOMEN ON SELF-SELECTED DIETS

MARY JONES HARP[1] AND FLORENCE I. SCOULAR
School of Home Economics, North Texas State College, Denton

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There has been a growing interest in cobalt since it was discovered to be a part of the B_{12} molecule. Harper and co-workers ('51) reported a significant increase in the B_{12} content of ewe milk when cobalt was added to the ration. Likewise Davis and Chow ('51) showed that the feeding of radioactive cobalt (Co^{60}) to rats increased the amount of radioactive B_{12} in their feces. These studies suggest that dietary cobalt may influence the production of B_{12} in human subjects. The present study was undertaken to determine the cobalt metabolism of young college women consuming self-selected diets.

PROCEDURE

Twenty-three young college women served as subjects while living in the Home Management House Duplex, North Texas State College. The technique used in the collecting of food and excreta for analysis in the present study was previously reported by Holt and Scoular ('48). The meals consumed during periods 1 and 2 were planned cooperatively by the two groups of young women residing in the duplex at the time. Each group, however, purchased and prepared its food separately. In a similar manner the meals were planned and prepared for periods 3 and 4. Milk was analyzed separately in order to permit each girl to consume the amount she desired. A sample of milk was taken from each day's supply

[1] Data submitted in partial fulfillment of the requirements for the Master of Science Degree in Foods and Nutrition, August, 1951.

in order to have one homogeneous sample for analysis for each period of the study. A record was kept of the amount of milk consumed by each girl each day since this was the only variable in the daily food intake. Suitable additions were then made to each individual's food intake to include the milk cobalt.

A serving of each food from each meal, similar in all respects to that eaten by the college women, was collected in weighed, aged glass jars at the same time that the subjects were served at the table. Only aged glassware was used in the collecting and storing of both food and excreta. The weight of the food was determined before each meal was macerated in a Waring Blendor. Aliquots of the day's meals were also mixed in the Blendor before analysis. The actual amount of tap water consumed by each girl was not considered in obtaining the total daily cobalt intakes because the subjects rarely consumed a liter of tap water as such, and one liter of tap water was found to contain not more than 0.1 μg of cobalt. This study was underway during hot weather when the use of cold beverages predominated over hot. Beverages were included in the analysis of composite food samples.

The menus followed a similar pattern each day: breakfasts consisted of fruit, breakfast meat or eggs, cereal or breakfast breads, preserves, and beverage; lunch contained a meat or meat substitute, salad, breadstuff, dessert, and beverage; while dinner contained a meat, two vegetables, salad, breadstuff, dessert, and beverage.

A compilation of the cobalt content of plant products by Young ('48) gives the cobalt content of plants consumed by man as varying from 1.20 mg/kg edible portion of spinach to 0.005 mg/kg for the edible portion of cherries. These values suggest that the amount of cobalt in self-selected diets would probably be small, and therefore a method which could determine minute amounts of cobalt was needed for our study. Two colorimetric methods were used in determining the cobalt in food, feces and urine during a preliminary study.

These methods were those of Yoe and Barton ('40) using beta-nitroso-alpha-naphthol, and Overholser and Yoe ('43) using ortho-nitrosoresorcinol. When recoveries of known amounts of cobalt were determined in food, feces, and urine by each of the two methods, it was found that the more consistent recoveries were obtained with the former method. Consequently, all the cobalt values reported for the present study were determined with the use of beta-nitroso-alpha-naphthol.

Duplicate 25-gm samples of both food and feces were used in obtaining the values reported. Additional samples were analyzed if the duplicates failed to check within 5%. The samples were weighed directly into 50-ml porcelain crucibles and ashed in an electric muffle at a temperature not exceeding 800°C. The 30-ml samples of pooled urine were also placed in 50-ml porcelain crucibles and evaporated to drynes in a hot air oven. Cobalt recoveries from food ranged from 90 to 110%; from urine, 92 to 109% and from feces, 91 to 108% during the 4 periods of this study. Neither the muffle nor the hot air furnace was used for other metal determinations during these periods. In fact, no other metal determinations were underway in the laboratory during the entire time and every precaution was taken to prevent contamination. Blanks were run on all the reagents used, including 1 l of distilled water evaporated to dryness, and no detectable amounts of cobalt were found. Therefore, no correction factor was used in calculating the determined amounts of cobalt in the aliquots used.

RESULTS AND DISCUSSION

The average daily intake and excretion of cobalt of these 23 young college women are given in table 1. The cobalt content of the day's menus was different for each of the 5 days, although only the average for the 5 days is shown in the table. During periods 1 and 2 the same menus were used but two different purchases of food were made and each group of girls used its own recipes. Consequently, the aver-

age daily amount of cobalt in the food was similar — 5.11 μg for period 1 and 5.63 for period 2. The weight of the daily composites during period 2 was slightly greater, suggesting slightly larger servings of the individual foods, which would account for the slightly higher cobalt values for the composite samples. Similar planning, purchasing and preparing

TABLE 1

Average daily cobalt intake and excretion of college women

SUBJECTS	COMPOSITE	INTAKE		EXCRETION			RETENTION	COBALT OF FOOD ABSORBED [1]
		Milk	Total	Urine	Feces	Total		
	μg	μg	μg	μg	μg	μg	μg	%
Period 1								
A.H.	5.11	2.17	7.28	0.43	1.21	1.64	5.64	83
B.L.	5.11	2.49	7.60	8.23	0.42	8.65	— 1.05	94
P.S.	5.11	1.19	6.30	3.72	0.46	4.18	2.12	93
P.W.	5.11	0.94	6.05	3.20	0.28	3.48	2.57	95
E.C.	5.11	1.22	6.33	6.58	0.49	7.07	— 0.74	92
V.Mc.	5.11	0.89	6.00	4.31	1.60	5.91	0.09	73
Period 2								
B.B.	5.63	2.29	7.92	3.52	0.46	3.98	3.94	94
B.H.	5.63	1.70	7.33	4.02	1.07	5.09	2.24	86
A.R.	5.63	0.00	5.63	5.35	0.26	5.61	0.02	95
F.S.	5.63	1.99	7.62	6.83	0.60	7.43	0.19	92
P.L.	5.63	2.34	7.97	5.81	0.40	6.21	1.76	95
Period 3								
E.B.	5.25	1.45	6.70	4.24	0.37	4.61	2.09	94
J.M.	5.25	1.45	6.70	3.43	0.39	3.82	2.88	94
M.M.	5.25	1.45	6.70	4.26	0.34	4.60	2.10	95
B.S.	5.25	1.45	6.70	3.99	0.48	4.47	2.23	93
K.W.	5.25	1.45	6.70	4.89	0.22	5.11	1.59	97
B.W.	5.25	1.45	6.70	3.77	0.20	3.97	2.73	97
Period 4								
P.B.	5.22	1.32	6.54	4.31	0.38	4.69	1.85	94
P.A.	5.22	1.10	6.32	3.73	0.19	3.92	2.40	97
P.E.	5.22	1.11	6.33	3.24	0.26	3.50	2.83	96
N.M.	5.22	1.32	6.54	5.71	0.42	6.13	0.41	94
R.P.	5.22	1.32	6.54	5.54	0.82	6.36	0.18	87
S.S.	5.22	1.75	6.97	5.72	0.47	6.19	0.78	93

[1] $\frac{\text{Food intake} - \text{Co in feces}}{\text{Food intake}} \times 100 = \%$ absorbed.

of the composite food occurred during periods 3 and 4. In the latter instance the weights of the daily composites were similar, with less variation in the cobalt content also — namely 5.25 and 5.22 μg.

The amount of cobalt from the milk consumed by the subjects varied from 0.00 to 2.49 μg per day. During only one period (period 3) was the daily milk consumption the same for all of the subjects, which accounts for the fact that not only was the milk cobalt the same but the total daily intake of cobalt was the same for the 6 subjects: namely, 6.70 μg. During the other three periods of the study the milk consumed by the subjects varied, so that the total daily intake of cobalt varied: namely, 5.63 for the non-milk drinker to 7.97 μg for one of the larger consumers of milk. Milk furnished from 0 to 33% of the total dietary cobalt.

The urine was the main pathway for excretion of cobalt. Approximately 67% of the cobalt intake appeared in the urine. This value is similar to that reported by Comar ('47), who found that 65% of the cobalt fed to animals appeared in their urine. The daily fecal cobalt was less than 1 μg for 20 of the 23 subjects. Furthermore, none of the larger fecal cobalt excretions was associated with a negative cobalt balance. There were just two negative balances, and in each case these balances were negative because of higher urinary cobalt; nor were the two negative balances due to an inability to absorb cobalt from the intestinal tract, since these subjects had absorption percentages of 92 and 94%, respectively, as compared with the range of 73 to 97% for the entire group of subjects.

The positive retentions ranged from 0.02 to 5.64 μg per day on total daily intakes of 5.63 to 7.79 μg of cobalt. During period 3, when the total daily intake was identical for the 6 subjects, the retentions varied less from subject to subject and were uniformly higher than during the periods when the daily intake varied from day to day and for each girl. On the basis of this study it appears that not only was the

dietary cobalt readily absorbed but that positive retentions were possible with these daily intakes.

Self-selected diets of 23 experimental subjects furnished remarkably similar cobalt values for the composite food samples: 5.11 to 5.63 μg. The total daily cobalt from composite foods and milk ranged from 5.63 to 7.79 μg.

SUMMARY

Twenty-one of the 23 subjects were in positive cobalt balance on these self-selected diets. From 73 to 97% of the dietary cobalt was absorbed.

The main pathway of excretion was through the urine, with an average of 67% of the total daily intake appearing in the urine.

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EFFECT OF VITAMIN B_{12} AND AMINO ACID IMBALANCES ON GROWTH AND LEVELS OF CERTAIN BLOOD CONSTITUENTS IN THE CHICK [1]

PENG-TUNG HSU [2] AND G. F. COMBS
Poultry Department, University of Maryland, College Park

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It has been shown that high levels of protein in vitamin B_{12}-deficient diets inhibit the rate of growth of chicks (Rubin and Bird, '47) and of rats (Cary et al., '46; Hartman et al., '49; Bosshardt et al., '50). Recently, Menge and Combs ('50) and Machlin et al. ('52) found that the growth of vitamin B_{12}-deficient chicks was depressed by the addition of high levels of glycine to their diets. Vitamin B_{12} supplementation largely overcame this growth depression.

The blood nonprotein nitrogen level was also found to be higher in chicks (McGinnis et al., '48; Charkey et al., '50; Hsu and Combs, '52) and in rats (Zucker and Zucker, '48) fed vitamin B_{12}-deficient diets than in controls which received the vitamin. Charkey et al. ('50) reported further that vitamin B_{12} was effective in reducing the blood levels of certain amino acids below those observed in vitamin B_{12}-deficient chicks. In addition, Hsu and Combs ('52) found that a vitamin B_{12} deficiency in chicks significantly increased the blood levels of amino nitrogen, urea nitrogen, creatinine, and glucose. Stern and McGinnis ('51) found that the toxicity which resulted from the administration of a 1-gm portion

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[2] Present address: Department of Physiological Chemistry and Nutrition, Institute for Cancer Research, Philadelphia, Pennsylvania.

of glycine by capsule to chicks was also alleviated by vitamin B_{12}. These workers further observed that the blood nonprotein nitrogen and amino nitrogen levels remained higher 4 hours following the administration of glycine in vitamin B_{12}-deficient chicks than in normal controls.

Since the above findings indicate that vitamin B_{12} is concerned in the metabolism of protein and amino acids, studies were conducted to determine the effect of this vitamin on the growth inhibition and mortality of chicks fed high levels of several different amino acids. Blood levels of glucose, nonprotein nitrogen, amino nitrogen, urea nitrogen, uric acid, and creatinine were determined. The results are reported herein.

EXPERIMENTAL

Eleven experiments were conducted with New Hampshire chicks of mixed sexes from dams maintained on wire floors. The dams were fed a vitamin B_{12}-deficient ration except as indicated. The chicks were maintained in batteries with raised wire floors and feed and water were supplied ad libitum. In all but three experiments, the chicks were fed the vitamin B_{12}-deficient basal ration during the first week, after which they were distributed into uniform experimental groups of from 16 to 20 chicks each. In the remaining experiments, day-old chicks were used. All experiments were terminated when the chicks were 4 weeks old. The average gain in body weight was recorded at weekly intervals.

The vitamin B_{12}-deficient basal diet used in the first 8 experiments had the following percentage composition: ground yellow corn 62.2; corn gluten meal 7.5; soybean oil meal (50% protein) 25; limestone 1.25; bone meal 3.25; iodized salt 0.5; A and D oil (3 I.C.U. of D and 2,250 I.U. of A per gram) 0.3; manganese sulfate 0.025; iodinated casein [3] 0.03. In addition, each pound of diet contained the following amounts of added vitamins (in milligrams): riboflavin 1.6; niacin 10; calcium pantothenate 5; choline chloride 250;

[3] Protamone, containing 3% thyroxine.

menadione 0.2; folic acid 0.35; biotin 0.045; and pyridoxine 1.6. The basal diet used in experiments 9, 10, and 11 was the same except that it contained somewhat higher levels of added vitamins, as follows (milligrams per pound): riboflavin 3; niacin 33; calcium pantothenate 5; choline chloride 250; menadione 0.2; folic acid 0.95; biotin 0.045; and pyridoxine 2.6. The percentage of crude protein and certain amino acids supplied by these diets was calculated to be as follows: crude protein 21.26; arginine 1.07; lysine 0.88; methionine 0.49; cystine 0.31; tryptophan 0.26; glycine 2.28; histidine 0.48; tyrosine 1.05; phenylalanine 1.20; threonine 0.83; leucine 2.81; isoleucine 0.94; and valine 0.92.

In these experiments, 9 different amino acids were fed at a level of 4%, as indicated. Zein also was used as a supplement in two experiments. In each trial, one group of chicks received the basal diet alone, while other groups were fed the basal diet plus single added amino acids or zein. Corresponding groups received the same diets plus supplementary vitamin B_{12}. In 4 trials, vitamin B_{12} was administered orally at a level of 3 μg per 100 gm of diet. In the remaining experiments, it was given by subcutaneous injections at weekly intervals. Each chick received 1.2, 1.8, and 2.4 μg of crystalline vitamin B_{12} on the 8th, 15th, and 22nd day of age, respectively. In the trials in which day-old chicks were used, 0.06 μg of crystalline vitamin B_{12} was also injected at the start of the study.

At the end of 6 experiments, the chicks were fasted for approximately 12 hours, and 2.5 ml of blood were removed by heart puncture from each of 8 chicks per group. The samples were pooled and analyzed for glucose, nonprotein, amino nitrogen, urea nitrogen, uric acid, and creatinine. Zinc sulfate and barium hydroxide were used to prepare the protein-free filtrate for the samples used in glucose determinations, while the tungstic acid method was employed for the remaining determinations. The method of Somogyi ('45) was used for the determination of glucose. The methods of Koch and McMeekin for nonprotein nitrogen; Brown, for uric

acid; Folin and Wu, for creatinine; and Danielson, for amino nitrogen, as modified by Hawk, Oser, and Summerson ('47), were also employed. Urea nitrogen was determined by the method outlined by Kibrick and Skupp ('50).

In two trials, glycine, methionine, and zein were also administered by capsule to vitamin B_{12}-deficient chicks and corresponding controls at the end of a 4-week feeding trial. After 12 hours of fasting in one trial, 0.5 gm of glycine or DL-methionine was force-fed to each chick, while in the other trial, 0.2 mg of glycine or zein was administered per 100 gm of body weight. Each chick which received glycine, methionine, or zein by capsule had previously received the basal diet plus 4% of the respective substances during the feeding trial. This procedure was adopted to observe further changes in the blood levels of the various constituents measured. Blood samples were removed at zero, two, and 4 hours after the administration of the capsule.

In two of these experiments, a glucose tolerance test was also performed following a 12-hour fasting period. Three grams of glucose per kilogram of body weight were administered orally as a 30% aqueous solution. A blood sample was taken just prior to the administration of glucose; another, one hour later; and a third, three hours later. Blood was withdrawn from 4 different chickens for each sample.

RESULTS

Effect of vitamin B_{12} and amino acid imbalances on chick growth

Excellent agreement was obtained among trials when diets containing excesses of individual amino acids were fed. Moreover, preliminary studies revealed that administration of vitamin B_{12} orally or by subcutaneous injection was equally effective in overcoming the growth inhibitory action of high levels of glycine. Accordingly, the results of these trials have been combined wherever possible for presentation.

A summary of the average gains and mortality obtained in these experiments is presented in table 1. The relative average gain for each group of chicks, expressed as a per-

TABLE 1

Effect of vitamin B_{12} and amino acid imbalances on chick growth and mortality

SUPPLEMENT (4% level)	NO. OF TRIALS	AVE. GAIN AND STANDARD ERROR DURING 3-WK. EXPTL. PERIOD (GM)		% MORTALITY	
		With vit. B_{12}	Without vit. B_{12}	With vit. B_{12}	Without vit. B_{12}
none	3 (54)[1]	208 ± 5	146 ± 8	5.6	46.3
L-leucine		200 ± 5 (96)[2]	110 ± 6 (75)[2]	1.9	25.9
none	5 (92)	223 ± 5	153 ± 6	3.3	29.3
Glycine		206 ± 5 (92)	121 ± 6 (79)	6.5	45.7
none	3 (53)	207 ± 5	136 ± 10	3.8	50.9
L-glutamic acid		193 ± 1 (93)	132 ± 18 (97)	1.9	43.4
none	3 (51)	214 ± 6	140 ± 10	2.0	45.1
DL-lysine		201 ± 9 (94)	140 ± 7 (100)	3.9	19.6
none	1 (18)	230 ± 6	145 ± 5	0	50.0
L-tyrosine		185 ± 10 (81)	108 ± 10 (74)	0	11.1
none	1 (18)	262 ± 6	128 ± 13	0	27.8
DL-aspartic acid		169 ± 7 (65)	76 ± 8 (59)	5.6	22.2
none	3 (51)	215 ± 6	152 ± 10	2.0	41.2
DL-alanine		150 ± 9 (70)	123 ± 11 (81)	13.7	39.2
none	3 (53)	213 ± 6	149 ± 8	1.9	43.4
L-cystine		147 ± 4 (69)	132 ± 6 (88)	9.4	39.6
none	1 (18)	262 ± 6	128 ± 13	0	27.8
DL-methionine		65 ± 8 (25)	52 ± 7 (41)	11.1	44.4

[1] Numbers in parentheses in this column refer to number of total chicks used per treatment.

[2] Numbers in parentheses in these columns represent the relative gain, expressed as a percentage of that obtained by the corresponding control chicks fed the same diet without the added amino acid.

centage of that obtained with corresponding control chicks fed the same diet without the added amino acid, is also given. The large differences in the average gain and mortality for comparable groups of chicks resulting from the administra-

tion of vitamin B_{12} reveal that a marked vitamin B_{12} deficiency existed.

These data clearly show that the addition of 4% L-leucine or 4% glycine to the diet inhibits the growth of vitamin B_{12}-deficient chicks. However, the growth inhibitory action of L-leucine was almost completely overcome by the administration of vitamin B_{12}. Likewise, vitamin B_{12} was almost as effective in overcoming the growth inhibitory action of glycine. The amino acids L-tyrosine and DL-aspartic acid also depressed chick growth when added singly at a level of 4% to the diet of vitamin B_{12}-deficient chicks, but the administration of vitamin B_{12} exerted only a slight effect in overcoming this growth depression. DL-alanine, DL-methionine, and to a lesser extent L-cystine, exerted growth inhibitory effects. The administration of vitamin B_{12} was of no value in alleviating this condition. L-glutamic acid or DL-lysine exerted little or no inhibitory action even when no vitamin B_{12} was supplied.

When vitamin B_{12} was supplied, the feeding of high levels of individual amino acids (except for DL-aspartic acid, DL-alanine, DL-methionine, and L-cystine) did not appreciably affect mortality. The addition of 4% DL-aspartic acid, DL-methionine, or glycine to the diet of vitamin B_{12}-deficient chicks increased the mortality, while the addition of L-glutamic acid, DL-alanine, or L-cystine had little effect. On the other hand, the addition of 4% L-leucine, DL-lysine, or L-tyrosine to the diet of similar chicks resulted in a decrease in mortality. The administration of vitamin B_{12} markedly reduced the mortality regardless of the amino acid levels of the diets.

Since leucine was found to exert a marked growth-inhibitory effect which could be overcome by vitamin B_{12}, two experiments were performed to determine the effect of zein on chick growth. Zein contains high levels of leucine and glutamic acid; therefore, the addition of zein to the diet would increase the levels of these amino acids fed. In experiment 8, zein was fed at a level of 6%, while in experiment 9,

8% zein was added. A second group of chicks received 4% added L-leucine, and a third received 4% added L-glutamic acid. Another group of chicks received 1.6% added L-leucine plus 2.4% added L-glutamic acid. These quantities of leucine and glutamic acid are comparable to those supplied by the addition of 8% zein. Control groups of chicks were fed the same diets but received vitamin B_{12} by injection.

TABLE 2

Effect of vitamin B_{12} and zein, leucine, and glutamic acid on chick growth and mortality

EXPERIMENT NUMBER [1]	SUPPLEMENT	AVE. GAIN AND STANDARD ERROR DURING EXPTL. PERIOD (GM)		% MORTALITY	
		With vit. B_{12}	Without vit. B_{12}	With vit. B_{12}	Without vit. B_{12}
8	none	245 ± 8	167 ± 16	0	16.7
	6% zein	238 ± 8 (97)[2]	125 ± 13 (75)[2]	0	22.2
9	none	240 ± 7	178 ± 29	5.3	63.2
	8% zein	226 ± 13 (94)	120 ± 15 (66)	5.3	68.4
	1.6% L-leucine + 2.4% L-glutamic acid	225 ± 9 (106)	154 ± 21 (87)	5.3	36.8
	4% L-glutamic acid	222 ± 6 (93)	177 ± 22 (100)	0	52.6
	4% L-leucine	232 ± 7 (97)	134 ± 16 (75)	0	36.8

[1] In experiment 8, vitamin B_{12}-deficient chicks were fed the vitamin B_{12}-deficient diet during a one-week preliminary period, after which they were distributed into uniform groups of 18 chicks each and maintained for three weeks on the experimental diets. In experiment 9, 19-day-old vitamin B_{12}-deficient chicks were used per group, and a 4-week experimental period was employed.

[2] Numbers in parentheses represent the relative gain, expressed as a percentage of that obtained by the corresponding control chicks fed the same diet without the added amino acid or zein.

The results obtained in these two experiments are given in table 2. The addition of either 6 or 8% zein to the diet of vitamin B_{12}-deficient chicks depressed growth to approximately the same extent as did the addition of 4% L-leucine. Likewise, the administration of vitamin B_{12} was effective in counteracting this growth inhibition. The addition of L-glutamic acid alone had only a very slight effect on growth

regardless of the amount of vitamin B_{12} supplied. However, when L-leucine and L-glutamic acid were fed to vitamin B_{12}-deficient chicks at levels comparable with that supplied by 8% zein, a slight growth depression again resulted, which was overcome by the administration of vitamin B_{12}. The imbalance produced by the addition of zein appears to be explained primarily on the basis of the increased leucine content of the diet. These findings indicate that the amino acid composition of the diet may greatly affect the vitamin B_{12} requirement of the growing chick.

In the last two trials, different levels of vitamin B_{12} were fed to chicks receiving diets containing no added amino acids and diets containing either 4% glycine or 4% L-leucine. The chicks used in experiments 10 and 11 were from vitamin B_{12}-depleted dams which had received 0.5 μg of vitamin B_{12} per pound of ration for a period of two and 4 weeks, respectively. The results are shown in table 3. The relative growth inhibition caused by the addition of either of these amino acids was progressively lessened as the vitamin B_{12} content of the diet was increased. Consequently, the vitamin B_{12} requirement was increased as a result of the added amino acid.

Effect of vitamin B_{12} and amino acid imbalances on certain blood constituents

The blood levels of nonprotein nitrogen, amino nitrogen, urea nitrogen, uric acid, creatinine, and glucose obtained in these experiments are shown in tables 4 and 5. In order to facilitate their presentation, the values obtained in different trials involving the same amino acid supplement have been averaged. Each of these values is also expressed as a relative percentage of the respective value for the corresponding controls which received an adequate intake of vitamin B_{12} with no supplemental amino acid or zein.

The differences between the blood values obtained for all groups of chicks receiving the vitamin B_{12}-deficient diet and those groups fed the same diet plus vitamin B_{12} were ana-

lyzed statistically, using the analysis of variance method. The blood nonprotein nitrogen and the amino nitrogen levels were higher in the vitamin B_{12}-deficient chicks than in those receiving vitamin B_{12}, the differences being statistically significant at the 1% level. The blood urea nitrogen, creatinine, and glucose levels were also higher in the vitamin B_{12}-deficient chicks. These differences were significant at the

TABLE 3

Effect of different levels of vitamin B_{12} on glycine and leucine toxicity

EXPERIMENT NUMBER [1]	VITAMIN B_{12} ADDED (μG PER KG OF DIET)	AVE. GAIN AND STANDARD ERROR DURING EXPTL. PERIOD (GM)		% MORTALITY	
		Without leucine	With 4% L-leucine	Without leucine	With 4% L-leucine
10	0	141 ± 22	112 ± 18 (79)[2]	55.6	16.7
	2	193 ± 8	167 ± 13 (87)	22.2	11.1
	5	189 ± 5	195 ± 6 (103)	22.2	0
	30	192 ± 7	198 ± 8 (103)	0	0
		Without glycine	With 4% glycine	Without glycine	With 4% glycine
11	0	172 ± 27	135 ± 16 (78)	35.7	42.9
	1	182 ± 13	161 ± 20 (88)	14.3	21.4
	5	243 ± 11	210 ± 10 (86)	21.4	0
	30	264 ± 7	241 ± 8 (91)	0	7.1

[1] Eighteen and 16 chicks per group were used in experiments 10 and 11, respectively. In experiment 11, the vitamin B_{12}-deficient diet was fed during a one-week preliminary period followed by a three-week experimental period. In experiment 10, day-old chicks and a 4-week experimental period were used.

[2] Numbers in parentheses in these columns represent the relative gain, expressed as a percentage of that obtained with the corresponding control chicks fed the same diet without the added amino acid.

5% level. No significant differences were observed in the blood uric acid level as a result of vitamin B_{12} supplementation. The increased gain in body weight resulting from vitamin B_{12} administration was also significant at the 1% level.

Similar increases were noted in the blood nonprotein nitrogen, amino nitrogen, urea nitrogen, creatinine, and glucose levels of vitamin B_{12}-deficient chicks fed diets containing an excess of individual added amino acids. The addition

TABLE 4

Effect of vitamin B_{12} and amino acid imbalances on nonprotein nitrogen, amino nitrogen, and creatinine of chicken blood

SUPPLEMENT	NO. OF TRIALS	MG %					
		Nonprotein nitrogen		Amino nitrogen		Creatinine	
		With B_{12}	Without B_{12}	With B_{12}	Without B_{12}	With B_{12}	Without B_{12}
None	4	36.1	45.8 (126.9)	20.9	22.6 (108.1)	0.54	0.77 (131.5)
4% glycine		40.5 (112.2)[1]	49.4 (136.8)	20.4 (97.6)	22.8 (109.1)	0.61 (113.0)	0.71 (131.5)
None	1	40.0	45.0 (112.5)	21.0	23.5 (111.9)	0.40	0.48 (120.0)
4% L-leucine	1	42.7 (106.8)	48.3 (120.8)	20.5 (97.6)	23.5 (111.9)	0.40 (100.0)	0.54 (135.0)
None	1	36.7	40.0 (109.0)	24.0	25.0 (104.2)	0.60	0.65 (108.3)
4% L-glutamic acid		37.6 (102.5)	42.7 (116.3)	23.5 (97.9)	24.0 (100.0)	0.56 (93.3)	0.60 (100.0)
None	2	38.9	43.0 (110.5)	22.0	23.0 (104.5)	0.55	0.73 (132.7)
4% DL-lysine		43.4 (111.6)	44.6 (114.7)	23.5 (106.8)	23.8 (108.2)	0.44 (80.0)	0.50 (90.9)
None	1	40.0	45.0 (112.5)	21.0	23.5 (111.9)	0.40	0.48 (120.0)
4% L-tyrosine		43.0 (107.5)	48.3 (120.8)	20.0 (95.2)	23.5 (111.9)	0.45 (112.5)	0.54 (135.0)
None	2	38.9	43.0 (110.5)	22.0	23.0 (104.5)	0.55	0.73 (132.7)
4% DL-alanine		40.0 (102.8)	47.0 (120.8)	22.3 (101.4)	24.8 (112.7)	0.60 (109.6)	0.60 (109.1)
None	2	40.5	45.5 (112.3)	20.5	22.3 (108.8)	0.45	0.64 (142.2)
4% L-cystine		42.0 (103.7)	44.0 (108.6)	20.3 (99.0)	21.5 (104.9)	0.45 (100.0)	0.52 (115.6)
None	1	37.5	47.0 (125.3)	20.5	22.0 (107.3)	0.50	0.62 (124.0)
4% DL-methionine		46.0 (122.7)	41.0 (109.3)	22.0 (107.3)	22.0 (107.3)	0.63 (126.0)	0.63 (126.0)
None	1	42.0	46.0 (109.5)	21.5	23.5 (109.3)	0.60	0.90 (150.0)
6% zein		43.0 (102.4)	49.0 (116.7)	22.0 (102.3)	24.0 (111.6)	0.76 (126.7)	0.80 (133.3)

[1] The values in parentheses represent relative percentages of the respective level for the corresponding control, and were obtained by assigning a value of 100% to the level of each blood constituent found in the groups of chicks receiving an adequate level of vitamin B_{12} and no supplemental amino acid or zein.

TABLE 5

Effect of vitamin B_{12} and amino acid imbalances on urea nitrogen, uric acid, and glucose of chicken blood

SUPPLEMENT	NO. OF TRIALS	MG %					
		Urea nitrogen		Uric acid		Glucose	
		With B_{12}	Without B_{12}	With B_{12}	Without B_{12}	With B_{12}	Without B_{12}
None	4	4.05	4.40 (108.6)	3.83	3.53 (92.2)	176.0	185.0 (105.1)
4% glycine		4.63 (114.3)[1]	4.76 (117.5)	3.80 (99.2)	3.68 (96.1)	189.0 (107.4)	195.0 (110.8)
None	1	8.10	9.25 (114.2)	1.85	1.85 (100.0)	185.0	190.0 (102.7)
4% L-leucine		9.00 (111.1)	10.50 (129.6)	2.30 (124.3)	1.80 (97.3)	185.0 (100.0)	195.0 (105.4)
None	1	6.00	9.00 (150.0)	2.40	2.60 (108.3)	172.0	192.5 (111.9)
4% L-glutamic acid		8.60 (143.3)	9.20 (153.3)	2.45 (102.1)	2.98 (124.2)	187.0 (108.7)	192.0 (111.6)
None	2	6.95	10.5 (151.1)	3.10	3.35 (108.1)	171.0	185.0 (108.2)
4% DL-lysine		10.00 (143.9)	10.5 (151.1)	3.23 (104.2)	3.20 (103.2)	165.0 (96.5)	178.8 (104.6)
None	1	8.10	9.25 (114.2)	1.85	1.85 (100.0)	185.0	190.0 (102.7)
4% L-tyrosine		9.25 (114.2)	11.50 (142.0)	2.40 (129.7)	2.65 (143.2)	182.5 (98.6)	185.0 (100.0)
None	2	6.95	10.50 (151.1)	3.10	3.35 (108.1)	171.0	185.0 (108.2)
4% DL-alanine		10.30 (148.2)	9.53 (136.7)	3.48 (112.3)	3.47 (111.9)	167.3 (97.8)	173.0 (101.2)
None	2	8.00	10.63 (132.8)	2.83	2.88 (101.7)	185.0	190.0 (102.7)
4% L-cystine		10.75 (134.3)	11.25 (140.6)	2.93 (103.5)	2.88 (101.7)	165.0 (89.2)	192.5 (104.1)
None	1	3.40	3.75 (110.3)	3.50	3.70 (105.7)	175.0	180.0 (102.9)
4% DL-methionine		4.25 (125.0)	4.25 (125.0)	3.40 (97.1)	4.05 (115.7)	202.0 (115.4)	185.0 (105.7)
None	1	15.75	18.50 (117.3)	6.00	4.20 (70.0)	175.0	180.0 (102.9)
6% zein		13.25 (84.1)	13.50 (85.7)	5.30 (88.3)	4.60 (76.6)	174.0 (99.4)	182.0 (104.0)

[1] The values in parentheses represent relative percentages of the respective level for the corresponding control, and were obtained by assigning a value of 100% to the level of each blood constituent found in the groups of chicks receiving an adequate level of vitamin B_{12} and no supplemental amino acid or zein.

of vitamin B_{12} to these diets reduced the blood levels of these compounds except when excess methionine was fed. Vitamin B_{12} also failed to decrease the level of urea nitrogen and creatinine when the chicks were fed a diet containing 4% DL-alanine.

The addition of single amino acids or zein consistently increased the blood nonprotein level in the presence of vitamin B_{12}. Increases were also noted when no vitamin B_{12} was given, except in chicks receiving 4% added L-cystine or DL-methionine. Despite the marked effect of vitamin B_{12} in lowering the abnormally high nonprotein nitrogen level of the blood in vitamin B_{12}-deficient chicks, the increases in nonprotein nitrogen which resulted from the additional amino acids fed were not appreciably affected by the addition of vitamin B_{12}. The amino nitrogen levels were little affected by the amino acid supplements.

The blood creatinine level of vitamin B_{12}-deficient chicks fed diets containing added DL-methionine, glycine, DL-alanine, or zein was no higher than that of similar chicks fed the unsupplemented basal diets. However, the addition of these amino acids or zein to the diet of chicks receiving adequate vitamin B_{12} increased the blood creatinine level. The feeding of high levels of L-glutamic acid and DL-lysine decreased the blood creatinine level regardless of the intake of vitamin B_{12}.

The level of blood urea was increased in almost all cases where the amino acid supplements were fed. The addition of zein, however, resulted in a decrease in the blood urea level both in the presence and absence of vitamin B_{12}. Only slight changes were observed in the levels of uric acid as a result of feeding high levels of amino acids.

The level of blood glucose of chicks receiving vitamin B_{12} was increased when glycine, or L-glutamic acid, or DL-methionine was added to their diets. The addition of 4% L-cystine, however, decreased the blood glucose level slightly in the presence of adequate vitamin B_{12}. The other amino acid supplements had little or no effect on the blood glucose level.

It was observed that vitamin B_{12}-deficient chicks consistently had higher blood glucose levels during glucose tolerance tests than chicks which received vitamin B_{12}. Moreover, vitamin B_{12}-deficient chicks which had previously received 4% DL-alanine, DL-lysine, L-tyrosine, and L-leucine had higher blood glucose levels at one and three hours following the administration of glucose than chicks fed the same diet with the addition of vitamin B_{12}. The blood glucose level of chicks which had previously received diets containing added DL-alanine or L-tyrosine and supplemental vitamin B_{12} remained slightly higher during the glucose tolerance test than the level in chicks not fed excessive amounts of these amino acids.

When capsules containing DL-methionine were given orally to 4-week-old vitamin B_{12}-deficient chickens previously fed a diet containing 4% added DL-methionine, increases in the blood levels of uric acid, nonprotein nitrogen, amino nitrogen, urea nitrogen, and glucose were observed 4 hours following the administration of the capsules. These changes in blood levels were also noted when vitamin B_{12} was supplied. However, a greater increase in the uric acid level as well as a slight increase in the level of creatinine were observed after two hours in chicks given vitamin B_{12} as compared with chicks not receiving this vitamin.

In the experiments where glycine and zein were administered in a similar manner, the effect of vitamin B_{12} on the levels of the various blood constituents two and 4 hours following administration of the capsules was very similar to that previously observed in the feeding trials. In all cases, the levels of nonprotein nitrogen, amino nitrogen, urea nitrogen, and glucose were somewhat higher in vitamin B_{12}-deficient chicks than in those receiving this vitamin. In general, the magnitude of the difference was little affected by the administration of the capsule during the 4-hour test period. An increase in the uric acid level did result from the administration of glycine or zein, which was more pronounced after two hours in the chicks receiving vitamin B_{12}.

When glycine was given, a slightly higher creatinine level was observed after two hours in chicks which had received vitamin B_{12} than in vitamin B_{12}-deficient chicks.

DISCUSSION

The growth inhibitory effect of 4% added glycine or L-leucine in vitamin B_{12}-deficient chicks confirms the observations of Menge and Combs ('50) and Machlin et al. ('52), who reported glycine toxicity in similar chicks. The fact that crystalline vitamin B_{12} was effective in overcoming this growth inhibition when administered by subcutaneous injection as well as by oral administration indicates that vitamin B_{12} is concerned in the metabolism of excess amounts of these amino acids. Since L-glutamic acid did not inhibit growth when fed at the same level, the apparent increase in the requirement for vitamin B_{12} cannot be explained on the basis of an increased nitrogen intake. The results of Menge and Combs ('50) also support this conclusion. These findings reveal that the levels of certain amino acids exert an influence on the dietary requirement for vitamin B_{12}.

Practical poultry rations which include large amounts of corn and soybean oil meal contain relatively high levels of both leucine and glycine. Since these amino acids increase the need for vitamin B_{12}, it appears that such rations should contain a higher level of this vitamin than is required in rations containing a more favorable amino acid balance. Excesses of certain other amino acids in the diet of vitamin B_{12}-deficient chicks also exerted growth inhibitory effects which were partially counteracted by vitamin B_{12}. Therefore, the levels of these amino acids also appear to influence the requirement for this vitamin. The growth inhibitory action of zein may be explained largely, if not entirely, on the basis of its leucine content.

This finding that the vitamin B_{12} requirement is influenced by the levels of certain amino acids in the diet is similar to that of Groschke and Briggs ('46), Groschke et al. ('48), and Anderson et al. ('51), who found the need for niacin to

be increased when chicks were fed a diet containing high levels of either glycine, alanine, leucine, arginine, aspartic acid, glutamic acid, or threonine. When these amino acids were fed singly at a level of 4% to chicks receiving a niacin-low diet, growth inhibition resulted which was overcome by the inclusion of niacin in the diet. These workers further demonstrated that the growth-depressing action of zein or gelatin in chicks fed niacin-low diets could be duplicated by feeding combinations of the amino acids which they contain.

The observation that a vitamin B_{12} deficiency results in an increase in blood nonprotein nitrogen, amino nitrogen, creatinine, urea, and glucose levels is in agreement with previous results obtained in this laboratory (Hsu and Combs, '52). It was also found that the addition of individual amino acids at a level of 4%, except for glutamic acid, resulted in growth depression. With few exceptions, increases in the nonprotein nitrogen and urea levels of the blood occurred at the same time. Administration of vitamin B_{12} to chicks fed diets containing high levels of single amino acids was also effective in reducing the blood levels of nonprotein nitrogen, amino nitrogen, urea nitrogen, creatinine, and glucose as compared with these levels in chicks not given vitamin B_{12}. Nevertheless, when vitamin B_{12} was administered, the blood levels of nonprotein nitrogen and urea in chicks fed the amino acid supplements were still higher than those in chicks not receiving the added amino acids.

Since the amino nitrogen level was not consistently increased as a result of feeding high levels of individual amino acids, either in the presence or in the absence of vitamin B_{12}, it appears that the amino nitrogen level of the blood was influenced more by the amount of vitamin B_{12} given than by the increased amount of the different amino acids fed.

Machlin et al. ('52) reported that the level of blood uric acid was usually increased by the administration of vitamin B_{12} to chicks fed a diet deficient in vitamin B_{12} and folic acid. They further reported that the inclusion of 6% additional

glycine in the diet also increased the blood uric acid level. In the present study, the administration of vitamin B_{12} to chicks fed diets containing high levels of glycine, L-leucine, or zein increased the amount of uric acid found in the blood as compared with that found in the blood of vitamin B_{12}-deficient chicks fed the same diet. Since these supplements also exhibited growth inhibitory effects which were overcome by vitamin B_{12} administration, these results suggest that vitamin B_{12} may be concerned in the formation of uric acid in the chick. The corresponding decrease in the amino nitrogen blood levels of chicks receiving high levels of leucine, glycine, and zein resulting from vitamin B_{12} administration lends support to this hypothesis. Machlin et al. ('52) have also suggested that vitamin B_{12} may function in this manner.

The very consistent increase in the blood glucose level of chicks resulting from a vitamin B_{12} deficiency suggests that vitamin B_{12} is involved in glucose utilization. This is also indicated by the higher blood sugar levels noted during the glucose tolerance tests in vitamin B_{12}-deficient chicks previously fed diets containing high levels of certain individual amino acids, as compared with those of chicks which received vitamin B_{12} and were fed the same diets. Also, the blood glucose levels of chicks fed adequate amounts of vitamin B_{12} were increased slightly more as a result of feeding high levels of glycine, glutamic acid, or methionine than they were in vitamin B_{12}-deficient chicks fed the same diet. This also suggests that vitamin B_{12} may function in the conversion of certain glucogenic amino acids into glucose. Furthermore, it has been reported by Bosshardt et al. ('50) that the source of energy in the diet exerts a marked effect on the requirement of mice for vitamin B_{12}. These workers found that the need for vitamin B_{12} was increased as the percentage of the total energy intake supplied by carbohydrate was raised at the expense of energy from fat. In similar studies with rats, McCollum and Chow ('50) obtained results which suggested that vitamin B_{12} is involved in the conversion of carbohydrate into fat.

SUMMARY

The growth of vitamin B_{12}-deficient chicks was inhibited by the addition of 4% L-leucine, 4% glycine, or 6 to 8% zein to their diet. The administration of vitamin B_{12} by subcutaneous injection or by oral administration counteracted this growth inhibition. The growth-depressing action of zein can be attributed to its leucine content. L-tyrosine and DL-aspartic acid fed at the same level exerted similar growth-inhibitory effects, although vitamin B_{12} was only partially effective in overcoming the growth depression. Excesses of DL-alanine, DL-methionine, or L-cystine also depressed growth, even when vitamin B_{12} was supplied. L-glutamic acid and DL-lysine, however, exerted only very slight growth-inhibitory effects.

The blood levels of nonprotein nitrogen, amino nitrogen, urea nitrogen, creatinine, and glucose in chicks were significantly elevated in a vitamin B_{12} deficiency. These differences were also generally observed in chicks receiving high levels of individual amino acids, except for DL-methionine. The addition to chick diets of different amino acids at a level of 4% resulted in an increase in the level of nonprotein nitrogen and urea nitrogen, with few exceptions. When vitamin B_{12} was administered to chicks fed diets containing high levels of glycine, L-leucine, or zein, the blood amino nitrogen was decreased, while the blood uric acid was correspondingly increased. The increase in blood glucose resulting from the feeding of 4% glycine, DL-methionine, or L-glutamic acid was greater when vitamin B_{12} was supplied. Possible ways in which vitamin B_{12} may function in metabolism are discussed.

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COMPARATIVE BIOLOGICAL ACTIVITY OF LEUCOVORIN AND PTEROYLGLUTAMIC ACID

HARRY P. BROQUIST, JOHN A. BROCKMAN, JR., MARVIN J. FAHRENBACH, E. L. R. STOKSTAD AND THOMAS H. JUKES

Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York

THREE FIGURES

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Recent investigations have shown (Broquist, Stokstad and Jukes, '51b; Brockman, Roth, Broquist, Hultquist, Smith, Fahrenbach, Cosulich, Parker, Stokstad and Jukes, '50) that leucovorin, a synthetic compound prepared by the reduction and formylation of pteroylglutamic acid (PGA), possesses biological properties similar to those of a factor in natural materials which is active for growth of *Leuconostoc citrovorum* and is termed the citrovorum factor (CF; Sauberlich and Baumann, '48). Chemical studies indicate that leucovorin, also termed "folinic acid S.F.," is 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (Roth, Hultquist, Fahrenbach, Cosulich, Broquist, Brockman, Smith, Parker, Stokstad and Jukes, '52; May, Bardos, Barger, Lansford, Ravel, Sutherland and Shive, '51; Pohland, Flynn, Jones and Shive, '51). In the present paper data are presented comparing the biological activities of leucovorin and PGA. A preliminary report of this work has appeared (Broquist, Stokstad and Jukes, '51a).

EXPERIMENTAL

Microbiological methods employing *Leuconostoc citrovorum* 8081 and *Streptococcus faecalis* 8043 and experimental pro-

cedures with mice and chicks have been described previously (Broquist, Stokstad and Jukes, '50). PGA deficiency was produced in turkeys using the method described elsewhere (Jukes, Stokstad and Belt, '47).

Lactobacillus casei 7469 was used for PGA assays following the procedure of Flynn et al. ('51). Leucovorin was synthesized as described elsewhere (Brockman et al., '50; Roth et al., '52). The crystalline free acid trihydrate was dissolved

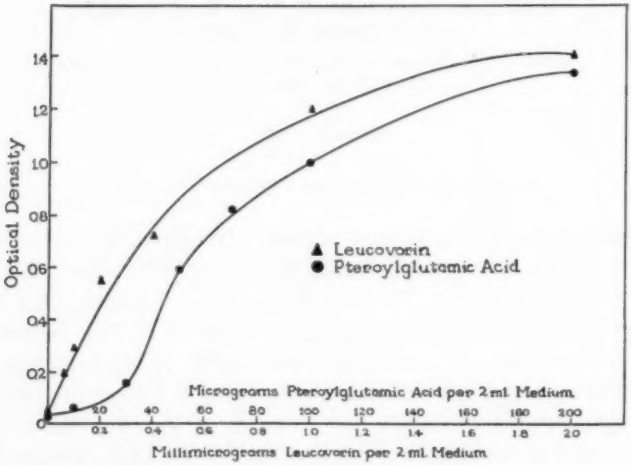

Fig. 1 The growth response of *Leuconostoc citrovorum* to leucovorin or pteroylglutamic acid.

in a small volume of 0.05 N NaOH, and precautions were always taken to keep the solutions at a pH greater than 7.0.

RESULTS

Growth response of microorganisms to leucovorin or pteroylglutamic acid

Typical growth responses of *Le. citrovorum* to either leucovorin or PGA are shown in figure 1. It was found from a number of microbiological assays that 0.15 mμg of leucovorin, as the anhydrous free acid, is required per milliliter

of culture medium to produce half-maximum growth of *Le. citrovorum* (table 1). About 200,000 times as much PGA is required by the organism for a comparable growth response. The responses of *Streptococcus faecalis* and *Lactobacillus casei* to leucovorin and PGA are also shown in table 1. For these organisms, leucovorin appeared to be somewhat less effective than PGA in promoting growth; thus approximately twice as much leucovorin as PGA was required to produce half-maximum growth.

TABLE 1

Comparative growth response of Leuconostoc citrovorum, Streptococcus faecalis and Lactobacillus casei to pteroylglutamic acid and leucovorin

ORGANISM	AMOUNT OF COMPOUND REQUIRED PER ML CULTURE MEDIUM FOR HALF-MAXIMUM GROWTH [1]	
	Pteroylglutamic acid	Leucovorin
	mμg	mμg
Le. citrovorum	30,000	0.15
S. faecalis	0.18	0.37
L. casei	0.09	0.17

[1] A 24-hour incubation period was used.

Growth responses of chicks and turkeys on folic acid-deficient diets to leucovorin or PGA

The response of folic acid-deficient chicks to leucovorin or PGA was next studied. When the supplements were administered by injection, leucovorin was approximately 50% as active on a weight basis as PGA in producing a growth response (table 2). In general the hematopoietic responses to the two substances paralleled the growth. The responses of the chicks to leucovorin were markedly affected by the route of administration of the vitamin. For example, 3 μg of PGA fed by pipette daily produced a growth response superior to that produced by 10 μg of leucovorin (experiment 4), but 10 μg of leucovorin were almost as effective as 6 μg of PGA when injected three times weekly (experiment 1).

Experiments were also carried out with turkeys (table 3), with results similar to those obtained with chicks. The tur-

keys were divided into groups of equal average weight after a depletion period of three days. A level of 15 μg of either PGA or leucovorin, when injected twice weekly, produced as good growth as 2 mg of PGA per kilogram of diet, but either 0.2 or 0.4 mg of PGA per kilogram of diet pro-

TABLE 2

Growth and hemopoietic responses of PGA-deficient chicks to pteroylglutamic acid and leucovorin [1]

EXP.	SUPPLEMENT	AMOUNT AND MODE OF ADMINISTRATION	RESULTS AT 28 DAYS		
			Av. wt.	Mortality	Hb
			gm	%	*gm%*
1	None		122	33	5.6
1	PGA	3 μg inj. 3 × weekly	184	11	6.6
1	PGA	6 μg inj. 3 × weekly	253	0	9.9
1	PGA	10 μg inj. 3 × weekly	291	11	10.5
1	Leucovorin	6 μg inj. 3 × weekly	201	11	9.1
1	Leucovorin	10 μg inj. 3 × weekly	229	0	10.0
1	Leucovorin	20 μg inj. 3 × weekly	306	0	10.7
1	PGA	5 mg per kg of diet	319	0	
2	None		111	33	
2	PGA	0.2 mg per kg of diet	225	27	
2	PGA	2.0 mg per kg of diet	324	0	
2	Leucovorin	1.0 mg per kg of diet	224	9	
2	Leucovorin	2.0 mg per kg of diet	259	9	
3	None		126	55	5.6
3	PGA	5 μg inj. 2 × weekly	206	0	6.3
3	PGA	15 μg inj. 2 × weekly	258	0	9.3
3	Leucovorin	5 μg inj. 2 × weekly	148	0	7.3
3	Leucovorin	15 μg inj. 2 × weekly	231	0	6.3
3	PGA	5 mg per kg of diet	280	0	8.0
4	None		140	90	4.8
4	PGA	2 μg inj. daily	202	0	5.8
4	PGA	5 μg inj. daily	287	0	7.8
4	Leucovorin	2 μg inj. daily	151	10	4.9
4	Leucovorin	5 μg inj. daily	252	0	7.3
4	PGA	3 μg orally by pipette daily	209	20	6.2
4	Leucovorin	3 μg orally by pipette daily	119	50	5.1
4	Leucovorin	10 μg orally by pipette daily	171	20	4.6
4	PGA	2 mg per kg of diet	286	0	8.9

[1] The chicks were fed the unsupplemented basal diet for a depletion period of 6 to 8 days, following which they were divided into groups of equal average weight before the test periods were started.

duced a better response than the same amounts of leucovorin, both in promoting growth and in preventing paralysis.

Growth inhibition studies with 4-aminopteroylglutamic acid (aminopterin)

Although the data in table 1 indicated that leucovorin was only half as effective as PGA in promoting growth of *S. faecalis,* if aminopterin, a folic acid antagonist, was incorporated in the medium, leucovorin was markedly more

TABLE 3

Response of turkeys on PGA-deficient diet to PGA or leucovorin (Twelve birds were used in each group)

SUPPLEMENT		ROUTE OF ADMINISTRATION	AVERAGE WEIGHT AND SURVIVORS (IN PARENTHESES) AT 28 DAYS	
PGA	Leucovorin		28 days	No. of cases of paralysis observed
..	..		274 (3)	4
15 μg	..	Injected 2 × weekly	353 (10)	0
..	15 μg	Injected 2 × weekly	353 (8)	0
0.2 mg	..	Added per kg of diet	311 (9)	0
0.4 mg	..	Added per kg of diet	332 (7)	0
2.0 mg	..	Added per kg of diet	327 (10)	0
..	0.2 mg	Added per kg of diet	268 (5)	5
..	0.4 mg	Added per kg of diet	271 (5)	4

effective than PGA in reversing the resulting growth inhibition (fig. 2). In the presence of equimolar amounts of leucovorin or PGA (0.0002 micromoles) which permitted maximum growth of *S. faecalis* in the absence of the antagonist, it was found that about 8 times as much aminopterin was required to reduce the growth of *S. faecalis* to half maximum in the presence of leucovorin as was needed in the presence of PGA.

The toxicity of aminopterin for mice, as for microorganisms, can be reversed by natural concentrates of CF (Broquist, Stokstad and Jukes, '50) and by leucovorin (Brockman

et al., '50). The effect of aminopterin on mice can be prevented by injecting approximately the same amount of leucovorin (group 4, table 4), but, as first shown by Burchenal, Babcock, Broquist and Jukes ('50), this proportionality exists only if the antagonist and metabolite are injected simultaneously, as is indicated in table 4. For example, leucovorin was markedly less effective in counteracting the toxicity of the antagonist if the metabolite was administered one hour after the aminopterin (group 5). It is apparent that

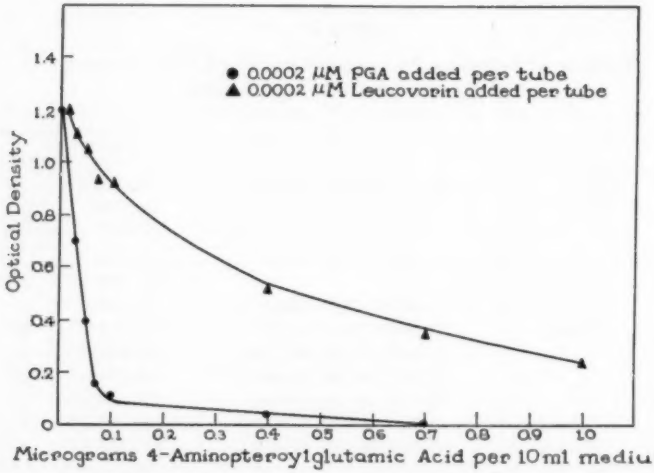

Fig. 2 Growth inhibition of *Streptococcus faecalis* by 4-aminopteroylglutamic acid in the presence of equimolar amounts of leucovorin or PGA.

the toxic effects of aminopterin are very rapidly established. When aminopterin was administered two hours preceding leucovorin, it was possible to reverse the toxicity by injecting 10 times as much leucovorin as was needed for protection when the substances were injected simultaneously (table 4, group 7).

DISCUSSION

In tables 1 and 2 it is seen that either leucovorin or PGA can effectively meet the requirement of *S. faecalis, L. casei*

and chicks for folic acid. Hill and Briggs have recently reported ('51) that synthetic "folinic acid" replaced PGA in the diet of the chick. *Le. citrovorum* is more exacting, in that it utilizes PGA only to an extremely limited extent but requires leucovorin. In the laboratory synthesis of leucovorin from pteroyl-L-glutamic acid, a new asymmetric center is formed at position 6 (fig. 3). Hence it is possible that leucovorin is a racemic mixture of diastereoisomers. This would explain our observation that leucovorin is about half as ac-

TABLE 4

Effect of time relationships on the reversal of aminopterin toxicity in mice by leucovorin

GROUP NO. (10 MICE PER GROUP)	SUPPLEMENT (INJECTED 3 × WEEKLY)	INJECTION OF LEUCOVORIN IN RELATION TO ANTAGONIST	WT. CHANGE AND SURVIVORS (IN PARENTHESES) AT 10 DAYS
1	No supplement		+ 4.5 (10)
2	10 μg aminopterin		— 3.5 (1)
3	10 μg aminopterin + 8 μg leucovorin	1 hour before antagonist	— 0.6 (9)
4	10 μg aminopterin + 8 μg leucovorin	Simultaneously	0
5	10 μg aminopterin + 8 μg leucovorin	1 hour after antagonist	— 2.4 (5)
6	10 μg aminopterin + 8 μg leucovorin	5 hours after antagonist	— 4.1 (6)
7	10 μg aminopterin + 80 μg leucovorin	2 hours after antagonist	+ 0.1 (10)

tive as PGA for *S. faecalis* and *L. casei* or when injected into chicks. It might also explain the finding by Keresztesy and Silverman ('51) that leucovorin is half as active as a crystalline natural CF obtained from horse liver when assayed with *Le. citrovorum* (Silverman and Keresztesy, '51). These authors also observed that the potency of leucovorin for *S. faecalis* was increased by treatment with dilute HCl, while the potency of "natural" CF was decreased. This observation may be explained on the following basis: the unnatural isomer of CF, which may be presumed to be biologically inactive and to represent half the weight of leuco-

vorin, would lose its asymmetric center at position 6 upon dehydrogenation of the tetrahydropyrazine ring and a biologically active product, such as 10-formyl PGA, could thus be produced by treatment with acid followed by exposure to oxygen.

Quantitative comparisons of leucovorin and PGA in pernicious anemia indicate that leucovorin is no more active than PGA and may be somewhat less effective in producing a reticulocyte response (Ellison, Wolfe, Lichtmann, Ginsberg and Watson, '51). These clinical findings parallel the present data obtained with chicks (table 2).

The smaller response of PGA-deficient chicks or turkeys to leucovorin when fed in the diet as compared to when it was injected (tables 2 and 3) may be compared with a uri-

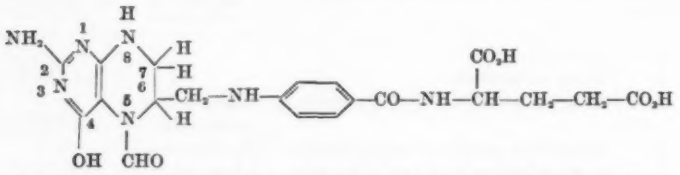

Fig. 3 Leucovorin, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.

nary excretion study in which it was found that when 3 mg leucovorin were injected into adult male human subjects, 22% of the dose could be recovered in the urine in 6 hours, but when the same amount of leucovorin was taken orally, only 1% of the dose could be recovered in the urine as CF (Broquist, Stokstad and Jukes, '51b).

Interest in the biological role that CF may play in metabolism is heightened by numerous studies indicating that leucovorin or concentrates of natural CF are more active than PGA in reversing the toxicity of folic acid antagonists in microorganisms (Broquist, Stokstad and Jukes, '50; Sauberlich, '49), mice (Broquist, Stokstad and Jukes, '50), rats (Nichol and Welch, '50), the chick embryo (Cravens and Snell, '50), *Drosophila* (Goldsmith and Harnly, '51), and human beings (Schoenbach, Greenspan and Colsky, '50).

The data in table 4 (compare groups 4, 5, and 7) showing that aminopterin toxicity in mice can be reversed by injecting an adequate amount of leucovorin are of interest in view of the work of Schoenbach, Greenspan and Colsky ('50), who demonstrated in two cancer patients that leucovorin was able to reverse some of the established toxic manifestations of aminopterin and 4-amino-10-methyl PGA ("A-methopterin").

The competitive reversal of the toxicity of aminopterin by leucovorin may be due to a direct competition between aminopterin and CF for essential reactions in living tissues. It has been suggested that aminopterin may be formylated and reduced *in vivo,* followed by condensation of the 5-CHO group with the 4-NH_2 group to give a stable compound which, in contrast to CF, could not participate in reactions in which the 5-CHO group could be reversibly transferred (Broquist, Fahrenbach, Brockman, Stokstad and Jukes, '51). This postulated "stable compound" could presumably displace CF from enzyme systems concerned in "single-carbon transfers." The greater effectiveness of leucovorin than of PGA in overcoming the toxicity of aminopterin has been related by Nichol and Welch ('50) to the effect of aminopterin in blocking the conversion of PGA to CF *in vivo.* It was noted (Broquist et al., '51) that tetrahydro-PGA was more effective than PGA in overcoming the toxicity of aminopterin, suggesting that the formation of CF from PGA *in vivo* is preceded by a reduction of PGA which may be blocked by aminopterin.

SUMMARY

1. The biological properties of leucovorin, a synthetic substance derived from pteroylglutamic acid (PGA) and having the biological properties of the citrovorum factors were compared with those of PGA. Leucovorin in the amount of 0.15 mμg per milliliter of culture medium was required for half-maximum growth of *Leuconostoc citrovorum,* but 200,000 times as much PGA was required to elicit the same growth response.

2. About twice as much leucovorin as PGA was needed for growth of *Streptococcus faecalis, Lactobacillus casei,* and chicks. Leucovorin was also effective in meeting the requirement of turkeys for folic acid. Leucovorin was more effective for chicks and turkeys when given by injection than when fed in the diet.

3. About 8 times as much 4-aminopteroylglutamic acid (aminopterin) was required to reduce the growth of *S. faecalis* to half maximum in the presence of leucovorin as was required in the presence of an equimolar amount of PGA.

4. With mice, approximately an equal amount of leucovorin was required to reverse the toxicity of aminopterin when the two substances were injected simultaneously. If the aminopterin dose preceded the leucovorin dose by one to two hours, the toxic effects could be reversed only by the injection of a much larger amount of leucovorin.

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RIBOFLAVIN IN HUMAN SERUM [1, 2]

K. SUVARNAKICH, G. V. MANN AND F. J. STARE
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

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During recent years it has become more and more evident that the signs and symptoms of riboflavin deficiency in man are not specific. Cheilosis, corneal vascularization, magenta tongue and seborrheic dermatitis, which have been associated with the clinical manifestations of human riboflavin deficiency, are not consistently present in riboflavin-deficient subjects and may result from one or many conditions other than riboflavin deficiency (Machella, '42; Machella and McDonald, '43).

Attempts have been made to correlate urinary riboflavin excretion with nutritional status. Among the various methods used are measurement of urinary riboflavin excretion in one hour during fasting (Holt and Najjar, '42; Johnson et al., '45a; Kark et al., '47), 24-hour urinary riboflavin (Brewer et al., '46; Davis et al., '46; Hou and Dju, '42), and load tests (Johnson et al., '45a; Goldsmith and Sarett, '48). The urinary riboflavin level indicating deficiency is, however, variable, and in the interpretation of these measurements there is no agreement between different workers (Johnson et al., '45b; Cogswell et al., '46; Goldsmith and Sarett, '48). The usefulness of load tests in evaluating riboflavin nutrition is also the subject of much controversy (Feder et al., '44; Hage-

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dorn et al., '45). Furthermore, riboflavin excretion has been reported to be influenced by both the recent food intake (Davis et al., '46) and the status of nitrogen balance (Sarett et al., '42; Oldham et al., '47). Riboflavin in blood has been measured by several procedures (Strong et al., '41; Bradford and Coke, '45; Lowry and Bessey, '44). Axelrod et al. ('41), using a microbiological procedure, found no difference in the riboflavin content of the blood of normal persons and of patients with riboflavin deficiency.

Burch, Bessey and Lowry ('48) have proposed a microfluorometric method for the determination of riboflavin in serum. The method is based on the following facts: there exist in serum three forms of riboflavin, namely, free riboflavin, flavin mononucleotide (in traces), and flavin-adenine-dinucleotide; in neutral solution and on a molecular basis, free riboflavin and flavin mononucleotide exhibit the same fluorescence, whereas flavin-adenine-dinucleotide is only about 14% as fluorescent; flavin-adenine-dinucleotide is completely hydrolyzed to flavin mononucleotide in 5% trichloroacetic acid after 20 hours at 37°C. In the sera of 13 normal adult human subjects these workers found an average free riboflavin plus flavin-mononucleotide of 0.8 μg % and total riboflavin of 3.2 μg %. They also found that serum total riboflavin in three human subjects decreased on a restricted riboflavin intake and increased immediately after a test dose. The increase in serum riboflavin level after a test dose was transitory (within an hour), in contrast to the prolonged increased output in the urine. In rats receiving diets containing graded amounts of riboflavin, these authors found that serum riboflavin diminished with decreasing riboflavin intake, and more so with free riboflavin than flavin-adenine-dinucleotide. They were of the opinion that serum riboflavin would be of diagnostic value in clinical medicine only if the levels in the serum reflect the nutritional status, as they seem to do in the rat.

Mann and Stevens ('50), using the same method of analysis, reported that plasma levels of riboflavin in 6 Cebus mon-

keys dropped to about 20% of normal after 6 weeks on riboflavin-deficient diets. All the animals lost weight but were otherwise apparently normal, except one showing seborrheic dermatitis over the dorsum of the hand and on the tail, with slight loss of hair. Riboflavin supplements induced a prompt gain of weight and a slower but steady return toward normal of the serum riboflavin levels (Mann et al., '52).

In view of the evidences that serum riboflavin levels respond to riboflavin restriction and supplementation in experimental animals, it is highly desirable that normal human serum riboflavin levels be investigated, in the hope that criteria for use in the diagnosis of human riboflavin deficiency may be obtained.

For the sake of simplicity, free riboflavin together with flavin mononucleotide will hereafter be called simply free riboflavin, since the latter is present in blood serum only in traces. The following abbreviations will also be employed: FR = free riboflavin; FAD = flavin-adenine-dinucleotide; TR = total riboflavin.

METHOD

The method used in this investigation is essentially the same as that described by Burch, Bessey and Lowry ('48). Some modifications, however, have been made to meet existing conditions.

The instrument employed for the measurement of fluorescence was the Farrand photoelectric fluorometer, which is equipped with a mercury vapor lamp. Corning glass filters No. 5113 and 3389 were used for incident light and No. 3486 for fluorescent light. This fluorometer requires 1.0 ml of solution in the cuvette instead of 0.5 ml as advocated by Burch, Bessey and Lowry. The fluorometer has been tested and found to show some deviation from a linear relationship between the galvanometer readings and the concentration of riboflavin. A standard curve has therefore been used which relates galvanometer deflections to riboflavin concentration.

Another modification has been made with respect to the volume of hydrosulfite solution used for reduction. Burch, Bessey and Lowry used 5 mm^3 of hydrosulfite solution for 0.5 ml of cuvette sample. In this investigation, it has been found more satisfactory to use 20 mm^3 of hydrosulfite solution for 1.0 ml of cuvette sample. The reduction has been found to be complete within two minutes after the addition of the hydrosulfite solution and to remain so for as long as 8 minutes.

MATERIALS

The serum specimens used in this investigation were obtained in laboratory personnel, industrial employees, and from urban hospitals. Hospital and clinic patients from whom sera were obtained were subjects afflicted with arteriosclerotic heart disease, hypertension or diabetes mellitus. The nutritional states of these patients, because of their continual medical attention, were presumed to be adequate. Therefore, all the subjects in this investigation can be classified under 4 groups, namely, (1) normal persons, (2) persons with coronary artery diseases, (3) persons with hypertension, and (4) persons with diabetes mellitus. Blood was drawn without respect to meals eaten or time of day. After clotting, the sera obtained were put in clean glass bottles, tightly stoppered and stored in a dark cold room at a temperature between 0 and 5°C. Serum riboflavin determinations were performed within 5 days after bleeding.

RESULTS

Variability of the method

The procedure for measurement of serum riboflavin was tested for its variability. A single sample of serum was obtained from a normal young adult woman. Sixteen riboflavin determinations were performed. The values averaged 2.16, 2.34 and 4.50 μg % for free riboflavin, flavin-adenine-dinucleotide and total riboflavin, respectively. The standard devia-

tions were 0.06, 0.08 and 0.06 μg %, and the coefficients of variation were 2.78, 3.42 and 1.33.

Effect of storage

The same sample of serum was stored in a dark cold room at a temperature between 0 and 5°C. Determinations of riboflavin were made on successive days for 4 weeks. The values obtained were essentially unchanged, indicating that serum stored under these conditions will retain its riboflavin content long enough to enable the investigator to perform the determination at his convenience during a reasonable length of time.

Variation of serum riboflavin levels within single individuals

Serum riboflavin levels in single individuals were determined at different dates. The individuals were on no diet restriction and were engaging in their usual activities. The daily variation of serum riboflavin levels under these conditions would be useful in any interpretation of cross sectional survey techniques. The data obtained from three subjects for a period of two to three weeks gave coefficients of variation for daily variation of 33% for free riboflavin and 8% for FAD. Serum free riboflavin is therefore more variable than serum FAD; the latter would be a better indicator of riboflavin nutriture.

Influence of riboflavin intake on serum riboflavin levels

Two normal subjects, after their serum riboflavin levels had been determined on several occasions, were asked to take 2.5 mg of riboflavin orally twice daily, in the morning and evening, for a period of 7 days. They were bled in the afternoon for serum riboflavin determinations. Serum free riboflavin levels in these two subjects during the period of riboflavin supplementation appeared consistently higher than

the average of those before the supplementation. However, when these figures were subjected to statistical analysis at a 5% level of "t," the differences between the two means — before and after supplementation — were not significant. Lesser changes were observed in serum FAD and were also not statistically significant. It has been shown by Burch and associates ('48) that the ingestion of riboflavin raises serum riboflavin levels temporarily within an hour of intake. Whether long-continued supplements in this experiment could have increased the fasting levels cannot at present be answered. It appears that they could, especially in the free riboflavin portion, but more prolonged experimentations are required to substantiate this point.

Influence of nitrogen balance on serum riboflavin levels

Three adult men were put on a protein-restricted diet of 1,200 cal. per day. Nitrogen determinations were performed on samples of the diet and on urine and feces in 48-hour periods; and nitrogen balance was computed as grams per 24-hour period. Serum riboflavin levels were determined at two-day intervals. The riboflavin contents of the diet were also determined. The daily riboflavin intake did not vary appreciably. The data are shown in table 1. When the deviations of serum riboflavin levels from the mean were plotted against nitrogen balance no apparent correlation was observed.

Normal levels

Serum riboflavin determinations were performed on 281 samples for this study. These were obtained from 183 males and 98 females. They were classified into 4 groups according to the state of health. The mean serum riboflavin values are shown in table 2. There was a statistically significant difference in free riboflavin values between the normal and diabetic groups, with the diabetic group showing a higher level. The FAD values of the cardiac and hypertensive groups were also

significantly higher than that of the normal group. It is therefore clear that for a definition of normal human serum riboflavin values the data should be taken from the non-diseased group only.

The serum riboflavin values of 141 persons in the normal group, together with their respective standard deviations and coefficients of variation, were calculated. The mean values were 0.84, 2.32 and 3.16 μg % for free riboflavin, FAD, and total riboflavin, respectively. These values are similar to those

TABLE 1

Serum riboflavin levels and nitrogen balance in three human subjects

Day	SUBJECT A			SUBJECT C			SUBJECT E		
	Nitrogen balance	Serum FR	Serum FAD	Nitrogen balance	Serum FR	Serum FAD	Nitrogen balance	Serum FR	Serum FAD
	gm/day	*μg%*	*μg%*	*gm/day*	*μg%*	*μg%*	*gm/day*	*μg%*	*μg%*
1	—3.4	0.96	2.80	—2.2	2.86	3.12	—9.4	1.23	3.32
3	—3.7			—2.6			—6.4		
5	—2.5	1.45	2.49	—1.3	2.70	2.74	—4.6	0.62	3.10
7	—2.2	2.51	1.78	—0.4	3.78	2.42	—2.6	0.41	3.90
9	—1.1	1.33	3.40	—0.3	1.61	4.36	—2.7	0.49	3.16
11	—0.5	1.57	3.16	—0.3	3.76	1.64	—0.8	0.79	3.21
13	—1.8	1.17	0.74	0.6	1.61	1.63	—0.5	0.33	1.58
15	0.1	1.61	2.92	—0.2	3.50	2.63	0.3	0.39	3.19
17	0.5	1.73	3.22	—2.6	2.66	2.93	—0.2	0.02	3.97
19		1.88	2.37		2.70	1.66	—0.4	0.28	3.27

found by Burch and associates ('48) for 13 normal humans. The standard deviations and coefficients of variation were 0.71, 0.42, 0.87 μg % and 84.5, 18.1, 27.5%, respectively, for the three components. The frequency distribution of the riboflavin values of the normal individuals is shown in table 3.

There were no significant differences in serum riboflavin values between the two sexes. These serum riboflavin values were also classified into age groups. The data are shown in table 4. There were no statistically significant differences between these age groups.

DISCUSSION

The serum riboflavin values obtained from 141 normal individuals were 0.84, 2.32 and 3.16 μg % for free riboflavin, FAD and total riboflavin, respectively. Similar values have been reported by Burch and associates ('48) in 13 normal subjects. There was no significant difference between the two sexes. Age was also not a factor causing differences in serum riboflavin values. The standard deviation for serum free riboflavin was 0.71 μg %, indicating a wide variation. The frequency distribution skewed to the right. These characteristics serve to indicate that serum free riboflavin is not a useful criterion for riboflavin nutriture.

TABLE 2

Serum riboflavin values of 281 well-nourished persons classified according to state of health

SUBJECTS	MALE				FEMALE				TOTAL			
	NO.	FR	FAD	TR	NO.	FR	FAD	TR	NO.	FR	FAD	TR
		μg%	μg%	μg%		μg%	μg%	μg%		μg%	μg%	μg%
Normal	82	0.81	2.31	3.17	59	0.89	2.26	3.15	141	0.84	2.32	3.16
Cardiac	67	0.82	2.57	3.39	21	1.03	2.42	3.45	88	0.87	2.53	3.40
Hypertensive	21	0.88	2.45	3.33	12	1.04	2.73	3.77	33	0.94	2.55	3.49
Diabetic	13	1.30	2.39	3.69	6	1.49	2.69	4.18	19	1.36	2.49	3.85
Total	183				98				281			
Mean		0.85	2.45	3.30		0.97	2.38	3.35		0.89	2.43	3.32
Standard dev.		0.80	0.49	0.93		0.87	0.46	1.06		0.81	0.48	0.99
Coef. of var.		94.1	20.0	28.2		89.7	19.3	31.6		91.0	19.8	29.8

The frequency distribution of serum FAD approximated the normal curve. Its standard deviation was 0.42 μg % and the coefficient of variation was 18.1. This greater stability of serum FAD would be of diagnostic value if it reflects nutritional status. Mann and Stevens ('50), and Mann et al. ('52) have reported a reduction in serum FAD levels in Cebus monkeys fed riboflavin-deficient diets. Burch and associates ('48) have reported a direct relationship between ribo-

TABLE 3

Frequency distribution of serum riboflavin values of 141 well-nourished normal persons

GROUP INTERVALS	MALE			FEMALE			TOTAL		
	FR	FAD	TR	FR	FAD	TR	FR	FAD	TR
μg%									
0.00–0.19	10			3			13		
0.20–0.39	13			8			21		
0.40–0.59	19			10			29		
0.60–0.79	13			12			25		
0.80–0.99	5			9			14		
1.00–1.19	8			6			14		
1.20–1.39	2	1		5			7	1	
1.40–1.59	3	3		1	1		4	4	
1.60–1.79	1	5	3	0	6		1	11	3
1.80–1.99	2	9	2	0	8	1	2	17	3
2.00–2.19	0	8	4	2	12	2	2	20	6
2.20–2.39	1	14	7	0	12	7	1	26	14
2.40–2.59	1	15	4	0	12	5	1	27	9
2.60–2.79	1	14	7	0	3	8	1	17	15
2.80–2.99	2	8	9	1	0	7	3	8	16
3.00–3.19	0	3	12	2	5	6	2	8	18
3.20–3.39	0	1	11			7	0	1	18
3.40–3.59	0	1	2			2	0	1	4
3.60–3.79	0		7			3	0		10
3.80–3.99	0		3			6	0		9
4.00–4.19	0		1			0	0		1
4.20–4.39	1		1			0	1		1
4.40–4.59			3			1			4
4.60–4.79			1			1			2
4.80–4.99			1			0			1
5.00–5.19			1			0			1
5.20–5.39			1			1			2
5.40–5.59			0			0			0
5.60–5.79			1			1			2
5.80–5.99			0			0			0
6.00–6.19			0			1			1
6.20–6.39			1						1
Total	82	82	82	59	59	59	141	141	141
Mean	0.81	2.31	3.17	0.89	2.26	3.15	0.84	2.32	3.16
Standard deviation	0.75	0.44	0.89	0.64	0.38	0.84	0.71	0.42	0.87
Coef. of variation	92.6	18.6	28.1	71.9	16.8	26.7	84.5	18.1	27.5

flavin intake and the levels of serum FAD in rats. These authors also found that total riboflavin in the serum of three human subjects dropped 8 to 25% from the initial levels after the riboflavin intake had been restricted to a level of about 0.4 mg per day for 4 days. However, the behavior of serum FAD was not mentioned. Animal experiments in this laboratory, including work with monkeys, dogs and ducks, invariably showed a decrease in serum FAD levels when the animals were restricted in or deprived of riboflavin intake, and a return to normal after adequate riboflavin supplementation. It may be assumed, unless proved otherwise by future experiments, that human serum FAD indi-

TABLE 4

Normal human serum riboflavin levels classified into age groups

AGE GROUPS	NUMBER	SERUM RIBOFLAVIN, μg%		
		FR	FAD	TR
Under 20	15	0.78	2.36	3.14
20–29	30	0.86	2.17	3.03
30–39	26	0.90	2.25	3.15
40–49	28	0.63	2.39	3.02
50–59	19	0.86	2.43	3.29
60 and over	8	1.18	2.74	3.92

cates riboflavin nutriture and is of diagnostic value in detecting riboflavin deficiency.

Total serum riboflavin is the sum of free riboflavin and FAD. As the FAD is the larger and more stable portion of the two, it follows that any marked reduction in total riboflavin must usually result from a reduction in FAD. Hence, a definitely low total serum riboflavin level can indicate riboflavin deficiency. This would be useful in a nutritional survey where laboratory facilities are limited.

The standard deviations for daily variation within single individuals (0.44 and 0.31 μg % for free riboflavin and FAD) were much larger than those for duplicate analysis (0.06 and 0.08 μg % for free riboflavin and FAD), indicating a small

error in the method of analysis in comparison to the wide variation that might be expected to be found in any single individual. For practical purposes, and provided the analysis is competently performed, duplicate determinations may be omitted without sacrificing the accuracy of the data.

Axelrod and associates ('41) reported that the concentrations of blood riboflavin in patients with clinical evidence of riboflavin deficiency were about the same as those of normal persons. They did not observe any decrease in blood riboflavin concentrations as the deficiency lesions became more severe. In their investigation, the microbiological method of assay was used. It is known that there are substances in the blood that may enhance or inhibit bacterial growth (Fraser et al., '40; Bradford and Coke, '45), and hence may interfere with the accuracy of microbiologic methods applied to blood. Furthermore, the riboflavin content of whole blood depends largely on the riboflavin contents of red and white cells. Burch and associates ('48) reported the average total riboflavin content of white blood cells plus platelets and of red cells from 12 human adults as 252 and 22.4 μg %, respectively. It can be easily seen that a slight change in the number of blood cells could easily obscure the behavior of serum total riboflavin.

The effect of daily riboflavin supplements has been studied, with no conclusive results. During 7 days of riboflavin supplements, serum riboflavin levels appeared higher than the initial level, but the difference was not statistically significant. The period of experimentation may not have been long enough to answer this question adequately.

Free serum riboflavin in diabetics, and serum FAD in persons with cardiac affections and hypertension, were observed to be higher than normal. These higher values cannot at present be explained. It may be that these persons were taking vitamin supplements regularly. Such long-term supplementation might cause a rise in the serum riboflavin levels, which, however, has not been definitely proved in the present investigation. One factor which was not determined is the

length of time between the last meal and the bleeding time. Burch and associates ('48) found that serum riboflavin rose within one hour after riboflavin intake. It is possible that the high serum riboflavin levels observed in this series of analyses were due to riboflavin intake which shortly preceded the bleeding. Since the majority of these subjects were hospital patients bled before breakfast, this explanation is improbable. These possibilities deserve further study.

Nitrogen balance, which has an influence on urinary riboflavin excretion (Sarett et al., '42; Oldham et al., '47), did not have a demonstrable effect on serum riboflavin levels. No correlation was observed between nitrogen balance and serum free riboflavin or FAD.

SUMMARY

The riboflavin content of normal human serum was investigated. In 141 normal well-nourished persons, free serum riboflavin and FAD were 0.84 and 2.32 μg %, with standard deviations of 0.71 and 0.42 μg %, respectively. There were no differences between sexes and age groups.

The microfluorometric method employed in this investigation gave an error much less than the variations that might be expected in any individual, hence it is suggested that duplicate determinations may be omitted in field survey techniques.

It was found that nitrogen balance and riboflavin intake had no appreciable influence on serum riboflavin levels, provided that bleeding were done several hours after riboflavin intake.

Serum riboflavin values, especially FAD, should be of value in the diagnosis of human riboflavin deficiency.

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THE GROWTH-PROMOTING EFFECT OF COMMERCIAL STRAINED MEAT AND FISH PRODUCTS INVESTIGATED WITH INFANTILE RATS

IHSAN EL RAWI AND E. GEIGER

Departments of Pharmacology and Biochemistry and Nutrition, University of Southern California, Los Angeles

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The introduction and commercial availability of strained meat products have revolutionized the theory and practice of infant feeding. Clinical experiences collected during nearly two decades have demonstrated that infants can digest and utilize such meat products at a very early age. It has been shown, furthermore, that calorically satisfactory diets, containing as their only protein source meat products supplemented with minerals and vitamins, can support normal growth and healthy development as well as milk can do so.

Commercially available strained meat products are derived from different animal sources such as beef, pork, veal, lamb and, more recently, fish. Where no allergies against some of these products are present, the choice in practical infant feeding is based mainly on taste preferences, because it is assumed that the nutritive value of the proteins present in such products is equivalent. This assumption is based mainly on the finding that the amino acid composition of beef, veal, pork and lamb is identical and very closely related to the composition of fish and milk products (Beach et al., '43; Dunn et al., '49; Neilands et al., '49).

It is, however, questionable whether the nutritive value is a function solely of the 10 essential amino acids. It is possible that the relative quantity of the other amino acids may

also be an important factor, especially in the nutrition of the fast-growing infant organism. Besides the amino acid content, the pattern according to which these compounds are found in the individual proteins may also influence the digestibility and utilization of the different meat products. We decided, therefore, to investigate the comparative biological value of the commercially available baby foods by determining their growth-promoting effects in experiments with infantile rats, using dried skim milk as the standard.

METHODS

Animals used

Male or female rats, Wistar strain, weighing 40 to 50 gm, were kept in individual false-bottom cages in air-conditioned animal rooms. Food consumption was determined daily; body weight every other day. The rats always had free access to water and to feed ad libitum, with the exception of the experiments where paired feeding was performed.

Analytical determinations of the composition of the strained food products

Total solids, ash and chloride were determined by the methods of the Association of Official Agricultural Chemists. Fat determination was performed by extraction of the dry, well-ground sample with ether for 6 hours in the Soxhlet apparatus; total nitrogen was determined according to the method of Kjeldahl; non-protein nitrogen was determined in the same way in the filtrate of the tungstic acid-precipitated sample. For the amide nitrogen determination the samples were hydrolyzed with 20% sulfuric acid for 6 hours at 100°C. After careful neutralization with sodium hydroxide, an excess of magnesium oxide was added and the liberated ammonia was steam-distilled into standard acid. The quanitity was determined by back titration of the acid. Amino acid and peptide nitrogen were determined after acid hydrolysis of the tungstic acid filtrate, by the formol titration method as modi-

fied by Melnick and Oser ('49). The nucleic acid nitrogen was calculated from the nucleic acid phosphorus content, which was determined according to the method of Schmidt and Thannhauser ('45). For the results, see table 1.

Preparation and composition of the diets

The strained meats used in the following experiments were dried *in vacuo* at 45 to 55°C. to the required moisture content.

TABLE 1

Composition of milk and strained meat products[1]

COMPOSITION IN PER CENT	BEEF	LAMB	PORK	TUNA	MILK
Total solids	19.7	20.6	21.0	21.5	96.5
Fat	4.8	6.1	5.4	0.48	0.05
Ash	1.05	1.12	0.68	1.00	7.96
Chloride as NaCl	0.31	0.36	0.29	0.300	1.77
Total nitrogen	2.2	2.06	2.37	3.04	5.67
Non-protein nitrogen	0.17	0.14	0.05	0.41	0.33
Amide nitrogen	0.13	0.13	0.14	0.13	0.53
Nucleic acid nitrogen	0.015	0.015	0.021	0.017	0.000
Amino nitrogen in protein-free filtrate	....	....	....	0.204	....

[1] The products used in these investigations were commercial strained meats and baby foods.

They were then mixed with the other ingredients of the diets, and, to insure homogeneity, the diets were passed through a sieve several times. The composition of the diets was equalized by making allowance for the fat and salt content of the meats and milk used. The ready-made diets were kept at approximately —20°C. and supplied daily after being brought to room temperature.

EXPERIMENTAL

Experiment 1

In the first group of experiments we compared the growth of rats on diets containing as the only protein source 6.7% protein derived from milk or strained meat products. The pro-

tein content was calculated by multiplying the total nitrogen by 6.25. A low protein supply was chosen as it was assumed that possible differences in the biological value of the different food mixtures may be more pronounced at such low levels. Because of the reported biological effects of lactose, this sugar was added to the different diets to make it equal in amount to the lactose content of the milk diet. The detailed composition of the diets is shown in the columns numbered one to 5 in table 2. Each diet was fed to 4 rats; the duration of the experiment was 19 days. The results of these experiments are shown in table 3 under experiment I.

The food consumption in the different groups varied to such a degree that a direct comparison of the growth promotion did not seem to be warranted. We calculated, however, for each group the "gain in gram per gram nitrogen consumed," and this value was, for the diets containing milk and tuna, nearly equal, and lower for the diets containing strained lamb, pork, or beef.

Experiment 2

Because in experiment 1 the food consumption on fish was higher than on beef, in the following experiment two groups of 6 rats each were pair-fed. For 21 days they received diets 6 (beef) or 7 (tuna), respectively. The results as shown in table 3 under experiment IIA indicate, in confirmation of the tentative results obtained in experiment I, that the absolute growth and the average gain per gram nitrogen consumed were somewhat lower on the beef than on the fish diets. The differences are statistically significant. Similar results were obtained in another experiment (IIB), where two groups of three rats each were pair-fed diets 1 and 4, respectively, for 13 days.

Experiment 3

In order to establish the reason for the observed differences in growth promotion, we investigated first whether different digestibilities of the proteins in the beef and fish diets

TABLE 2

Composition of diets

DIET NO.	1	2	3	4	5	6	7
Beef baby food	437 (186)					510 (105)	
Lamb baby food		466 (196)[4]					
Pork baby food			424 (189)				
Tuna baby food				315 (172)			372 (85)
Skim milk powder					169		
Cottonseed oil	79	72	78	99	100		
Butterfat						51.3	51.3
Salt mixture A [1]	34	35	36	37	27	51.3	51.3
Salt mixture B [1]							
Ammonium citrate							
Cod liver oil	5	5	5	5	5	12.7	12.7
Vitamin soln.–I [2]							
Vitamin soln.–II [2]	10	10	10	10	10		
Rice bran concentrate [3]						103	103
Calcium gluconate							
Potassium acid phosphate							
Magnesium citrate							
Water					94		
Cornstarch — to make	1 kg	1 kg	1 kg	1 kg	1 kg	1 kg	1 kg
Per cent moisture	10	10	10	10	10	..	..
Protein, per cent of dry ingredients	6.7	6.7	6.7	6.7	6.7	7.0	7.0

DIET NO.	8	9	10	11	12	13	14
Beef baby food	728 (364)			680 (238)			
Lamb baby food					715 (248)		
Pork baby food							
Tuna baby food		528 (315)				442 (195)	
Skim milk powder			283				267
Cottonseed oil				47.4	33.8	77.7	80.0
Butterfat	65	97	100				
Salt mixture A [1]				22.8	20.5	24.6	8.8
Salt mixture B [1]	10	10	10				
Ammonium citrate				4.0	4.8	15.4	2.4
Cod liver oil	5	5	5	5	5	5	5
Vitamin soln.–I [2]	10	10	10	10	10	10	10
Vitamin soln.–II [2]							
Rice bran concentrate [3]							
Calcium gluconate	66	66					
Potassium acid phosphate	23.0	22.2					
Magnesium citrate	4.0						
Water			190				
Cornstarch — to make	1 kg	1 kg	1 kg	1 kg	1 kg	1 kg	1 kg
Per cent moisture	22	20	20	10.4	10.0	10.0	10.0
Protein, per cent of dry ingredients	12.9	12.8	12.8	10.7	10.7	10.7	10.7

[1] *Salt mixtures:*
A — Osborne and Mendel formula.
B — Ferric citrate 15 gm, sodium fluoride 0.1 gm, magnesium sulfate 0.15 gm, manganese sulfate 0.15 gm, copper sulfate 0.5 gm, sodium chloride 500 gm.

[2] *Vitamin solutions:*
I — Thiamine 12.5 mg, riboflavin 25 mg, pyridoxine 12.5 mg, niacin 50 mg, inositol 0.5 gm, calcium pantothenate 0.1 gm, folic acid 5 mg, biotin 2.5 mg, choline chloride 5 gm, Reticulogen 3 ml, water to make 100 ml.
II — Same as (I) but without the Reticulogen. Three milligrams α-tocopherol in cottonseed oil supplied weekly to every rat.

[3] Fortified with 18 mg riboflavin, 40 mg calcium pantothenate, and 0.9 gm choline chloride per kilogram diet.

[4] Numbers in parentheses refer to weight of food after drying.

TABLE 3

Growth of rats on strained meat and milk products

EXPERIMENT NO.	WEIGHT OF RATS AT START	DIET NO.	PROTEIN IN DRY DIET	AVERAGE GAIN PER RAT	DURATION OF EXPERIMENT	AVERAGE CONSUMPTION	STANDARD DEVIATION OF MEAN	AVERAGE GAIN PER GM N CONSUMED	NUMBER OF RATS
	gm		%	*gm*	*days*	*gm*		*gm*	
I	47–48	1 (beef)	6.7	27.8	19	199		14.5	4
		2 (lamb)	6.7	32.8		195		17.5	4
		3 (pork)	6.7	32.0		196		17.0	4
		4 (tuna)	6.7	38.5		210		19.1	4
		5 (milk)	6.7	36.0		195		19.2	4
II–A	43–45	6 (beef)	7.0	22.5	21	168	1.7	12.9	6
		7 (tuna)	7.0	33.0		170	1.6	18.5	6
II–B	54	1 (beef)	6.7	12.7	13	94.6		13.9	3
		4 (tuna)	6.7	17.3		94.6		19.1	3
IV	42–44	11 (beef)	10.7	60.4	19	232		16.9	8
		12 (lamb)	10.7	61.4		240		16.6	5
		13 (tuna)	10.7	54.2		194		17.7	10
		14 (milk)	10.7	61.0		200		19.8	9
VI–A	52–54	(beef)[1]	18.8	46.6	19	286	3.9	10.2	5
		(tuna)[1]	18.8	69.6		298	2.2	15.0	5
		(milk)[1]	18.8	67.6		261	1.2	16.8	5
VI–B	50–52	8 (beef)	12.8	41.5	21	205	5.2	12.6	4
		9 (tuna)	12.8	51.8		244.8	7.3	13.3	4
		10 (milk)	12.8	72.8		228.5	4.9	19.9	4

[1] For composition of diets in this group, see McQuarrie and Ziegler ('50).

might be the responsible factor. For this purpose the feces of the rats used in experiment IIB were collected for the last 5 days and the nitrogen content of the dried feces was determined. Table 4 shows that the apparent digestibilities of the protein present in the beef and tuna diets respectively were nearly identical. The digestibility of beef was somewhat lower. The difference, however, was not sufficient to explain the observed differences in growth promotion.

Experiment 4

A possible reason for the different growth-promoting properties of the diets used has been provided by earier experiments in which baby chickens and turkeys showed better growth on fish scrap than on meat scrap (Agricultural Research Administration, '48). Subsequent investigations indicated that the growth factor present in fish meal may be identical with vitamin B_{12}. We performed, therefore, the following feeding experiments in which the growth factor vitamin B_{12}, in the form of Reticulogen [1] was added in excess of the requirements (see diets 11 through 14).

TABLE 4

Digestibility of the protein in beef and tuna diets

CATEGORY OF INTEREST	BEEF	TUNA
Nitrogen of food consumed — 5 days	1,538 mg	1,542 mg
Nitrogen of feces collected in 5 days	216 mg	178 mg
Apparent digestibility [1]	86.0%	88.5%

[1] Apparent digestibility $= \frac{\text{N of food} - \text{N of feces}}{\text{N of food}} \times 100.$

In this experiment the rats consumed less of the milk and fish diets than of the beef and lamb diets (see table 3, experiment IV). The absolute growth gains on these diets, therefore, cannot be compared directly. The differences in the "average gain per gram of nitrogen consumed," however,

[1] Lilly.

were similar to those in the other experiments. The average gain was highest for the milk diet, somewhat lower for fish, and beef and lamb followed in order.

The value for the beef diet in this experiment was higher than in the previous ones and approached closely the value for the fish diet, indicating that the addition of Reticulogen (B_{12}) improved the utilization of the beef diet.

Because of the large differences in food consumption observed in the previous experiment, the B_{12}-supplemented beef and fish diets were pair-fed to another group of rats.

Experiment 5

Table 5 shows that in this experiment, with the exception of pair No. 6, the growth on the fish diet was consistently higher than that on the beef diet. The average gain per gram of nitrogen consumed was similar to that in experiment IV; i.e., on the average, lower for beef than for fish, but higher than in the previous experiments in which no B_{12} was added to the diet.

Experiment 6

McQuarrie and Ziegler ('50) reported recently that supplementation of diets containing strained beef with calcium and phosphorus up to the level of these nutrients contained in the milk diet equalized the nutritive value of those diets. In order to find out whether a different content of these mineral constituents might be one of the factors responsible for the differences observed in our present experiments, we prepared diets from milk, strained beef and strained fish according to the data published by McQuarrie and Ziegler. These diets were fed to 5 rats each for 19 days. Experiment VIA in table 3 shows that in spite of equalizing the mineral content of the diets, the growth-promoting activity of the milk was best; next followed strained tuna and then beef.

The average gain per gram nitrogen consumed in this experiment was conspicuously lower for milk than in all the other experiments, probably because of the unsatisfactory

vitamin supplementation of the diets in this group. In order to establish more nearly comparable conditions, we repeated the experiments, using vitamin supplements which are more adequate than those used in McQuarrie and Ziegler's experiment. The mineral content of these diets also was equalized with that of the milk diet (see columns numbered 8, 9 and 10,

TABLE 5

Experiment with paired feeding

PAIR NO.	SEX	DIET	STARTING WEIGHT	FINAL WEIGHT	GAIN	EXCESS GROWTH ON TUNA	FOOD CONSUMPTION	GAIN PER GM NITROGEN CONSUMED
			gm	*gm*	*gm*	*gm*	*gm*	
1	F	Beef	43	75	32		131	15.7
		Tuna	43	86	43	11	131	21.4
2	F	Beef	40	76	36		134	17.5
		Tuna	40	81	41	5	134	19.9
3	M	Beef	42	70	28		127	14.4
		Tuna	42	76	34	6	127	17.45
4	M	Beef	39	74	35		137	16.0
		Tuna	39	79	40	5	137	19.1
5	F	Beef	35	63	28		116	15.45
		Tuna	35	66	31	3	116	17.5
6	M	Beef	42	84	42		132	20.7
		Tuna	42	84	42	0	132	20.7
7	M	Beef	41	70*	29		121	15.6
		Tuna	41	76	35	6	121	18.9

in table 2). These diets were fed to three groups of 4 rats each for 21 days. Experiment VIB in table 3 shows that in these experiments, similar to those discussed before, milk again had the best growth-promoting property; then tuna and beef followed in order. We do not know which factor may be responsible for the differences between the results of our experiment and those of McQuarrie and Ziegler. Our ex-

periment shows, however, that equalization of the mineral content itself does not bring up the growth-promoting value of the meat and fish diets to that of milk.

DISCUSSION

The experiments discussed in the foregoing show that the commercially available strained meat and fish products investigated promoted satisfactorily the growth of infantile rats even when fed at a low protein concentration of 6.7% of the total diet. The results of the experiments reported show also that the average gain per gram of nitrogen consumed was highest for the groups receiving skim milk powder in their diets. The second-best growth occurred with diets containing strained tuna. The growth for the groups on strained meat products was somewhat lower.

Evaluation of the experiments with ad libitum feeding is complicated by the considerable differences in food consumption. We are not yet able to ascertain which of the factors, such as taste differences and slight variations in consistency, particle size, and so forth, may have been responsible for the preference for some diets. In spite of the unequal food consumption, the differences in average growth per gram of nitrogen consumed were in all experiments of the same order. We proved further, with paired feeding, that the differences observed were significant.

Whether the differences observed using normal rats may be of practical significance with respect to the feeding of infants has yet to be investigated.

In discussing the possible causes of the observed differences, we must first consider the different biological values of the proteins investigated, because it is usually assumed that the growth-promoting value of meat and fish products is determined primarily by the "quality" of the proteins present. According to the literature (Kraybill, '48), the meat proteins present in the diets investigated by us were of high biological value. The literature on the nutritional value of fish protein has been recently reviewed (Geiger, '48), with

the conclusion that "fish protein has a high biological value which is probably somewhat above that of beef."

It is further assumed that the biological value of a protein is determined by the constituent amino acids. The possibility that a difference in the amino acid composition may be responsible for the observed differences seems to be questionable because, according to several authors (Beach et al., '43; Dunn et al., '49; Neilands et al., '49), "the muscle tissue of . . . different classes of animals does not differ vitally . . . and indicates that as far as these 10 amino acids are concerned, the protein of one muscle is as good as that of another supplying amino acids in the diet."

It should be emphasized, however, that such statements are based on the assumption that the nutritive value of a protein depends only on the quantities of the 10 essential amino acids present. We have to consider, however, that we do not yet know how the nonessential amino acids influence the digestibility and utilization of the protein. But even a total inventory of the essential and nonessential amino acids could not reveal those structural differences which are responsible, for instance, for the immune biological properties of the different proteins and which may very well influence also the gastrointestinal liberation and utilization of the amino acids present in proteins.

Differences in the protein composition of mammalian and fish muscle were discussed by Geiger ('48) and, more recently, by Hamoir ('51).

The posssibility that differences in digestibility may be responsible for the observed results could not be supported by our experiments. We found, in agreement with Nilson et al. ('47), that the digestibility of fish nearly equals that of beef. These investigations, however, do not reveal differences in the availability of the individual amino acids. Such differences are suggested by the experiments of Mayfield and Hedrick ('49), who found that canning reduces the biological value of beef protein by decreasing the availability of lysine. The experiments of March et al. ('50) suggest fur-

ther that the biological value of fish protein is less affected by heat treatment than that of meat protein.

It should also be emphasized that in the present experiments we did not investigate the purified protein fraction, but the strained meat product itself. Such experiments may therefore be complicated further by the presence of nitrogen-containing non-protein compounds. Table 1, for instance, shows that about 13½% of the total nitrogen in strained tuna is present in the non-protein fraction. In contradistinction, strained meat contains only 7 to 8%, and milk only 5.8%, of the total nitrogen as non-protein nitrogen. These data agree very well with those published recently by Shewan ('51), who found, in fish belonging to the clupeids, that 14 to 18% of the total nitrogen was in non-protein form. It is therefore possible that compounds present in the non-protein nitrogen fraction which may be lost during the isolation and purification of proteins can also influence the growth-promoting value of such complex foods.

In the experiments presently reported, we disregarded the differences in non-protein nitrogen content of the different meat products and prepared our diets on the basis of total nitrogen content. As the non-protein nitrogen content of strained tuna was found to be the highest, we fed, in the diet containing fish, actually less protein than in the meat diets. In spite of this difference, the "protein efficiency" of the diets containing fish was constantly somewhat higher than that of the diets containing meat products and somewhat lower than that of the diets containing milk.

That factors other than proteins may be responsible for differences in growth promotion has been suggested by the report of the Chief of Animal Industry of the Agricultural Research Administration ('48), who found that: "Turkeys did not grow as well when fed meat meal from hatching time as if changed from fish to meat meal when four weeks of age. Experiments with chickens showed that meat meal contains considerably less of the unknown vitamin than does fish

meal." This unknown factor was later tentatively identified as vitamin B_{12}.

Experiments IV and V, in which vitamin B_{12} was added to the diets in excess of the requirements, show that addition of this growth factor did not completely eliminate the difference in growth promotion observed in the earlier experiments. The difference between the average gains per gram of nitrogen consumed on beef and on fish diets was somewhat smaller than in the earlier experiments, but still present. These experiments support the assumption that fish meat may contain some growth factors in addition to vitamin B_{12}.

The results so far indicate that in studies on the nutritive value of protein foods such as strained meat products, the non-caloric effect of the non-protein food constituents must also be considered, in addition to the amino acid composition and the digestibility of the protein (Geiger, '51). We intend to investigate further which of the other factors present in the diets might be responsible for the differences in growth promotion observed in the present experiments.

SUMMARY

The growth-promoting effect of diets containing, as the only protein source, commercial strained meat or fish products has been investigated, using as controls diets containing dried skim milk powder. All of the investigated products promoted the growth of rats, even when fed at a low protein concentration of 6.7% of the total diet.

The average gain per gram of nitrogen consumed was in all experiments the highest for the group receiving in its diet skim milk powder. The second best growth was observed in the group receiving protein derived from strained tuna. The average growth for the groups on strained meat products was somewhat lower. Equalization of the mineral content up to the level in the milk diet did not eliminate the difference in growth promotion. Addition of B_{12} (Reticulogen) improved the utilization of the beef diet.

These experiments show that in studies on the nutritive value of complex protein foods such as strained meat products, the non-caloric effect of the non-protein food constituents will have to be investigated further.

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THE DETERMINATION OF THE PROTEIN REQUIREMENT OF THE RAT FOR MAXIMUM GROWTH UNDER CONDITIONS OF RESTRICTED CONSUMPTION OF FOOD

H. H. MITCHELL AND JESSIE R. BEADLES[1]
Division of Animal Nutrition, University of Illinois, Urbana

TWO FIGURES

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Since the paired-feeding procedure in nutrition experiments was first proposed by Armsby ('21) for the determination of the protein requirements for the growth of cattle, many other investigators have used this, or some other, method of controlled feeding inevitably involving a restriction of food consumption and of growth. To name only a few reported investigations of this nature, attention may be called to the work of Dunlop ('35) and of Aubel and associates ('36, '41) on the mineral requirements of swine, of Watkins and Mitchell ('36) on the phosphorus requirements of growing chickens, of Robinson ('43) on the thiamine requirement of the growing rat, of Mitchell and associates ('50) on the riboflavin requirement of the growing pig, of Snyderman et al. ('49) on the choline requirement of the baby pig, and of Brinegar et al. ('50a) on the lysine requirement and ('50b) on the isoleucine requirement for the growth of swine.

Although in these experiments restricted growth resulted from controlled feeding, no apprehension was expressed that the significance of the nutrient requirements thus obtained

[1] Deceased.

was impaired in the sense that such requirements would not induce maximum growth. Even in experiments involving ad libitum feeding and concerned with the quantitative evaluation of nutrient requirements there is no assurance that maximum growth was in fact realized, and in many cases it is evident that it was not attained.

The implication in all of these experiments, with controlled or uncontrolled feeding procedures, is that the rate of food consumption and the rate of growth resulting in the experimental animals do not determine the level of the indicated nutrient requirements, and that such requirements need not be qualified, or restricted in their application to practical feeding conditions by the growth realized experimentally.

It will be the purpose of this report to subject this proposition to experimental enquiry, using the growing rat as the experimental animal. The results obtained justify the proposition, within certain limits, but for reasons that may not be obvious on first thought.

EXPERIMENTAL PROCEDURE

The experiment was set up to measure the effect of different levels of food consumption on the requirement for a definite protein by growing rats as indicated by the growth responses. A series of diets was prepared containing defatted, dehydrated (laboratory-prepared) whole egg in proportions to provide 8, 10, 12, 14, 16, 18 and 20% protein ($N \times 6.25$). The diets all contained 4% of salt mixture and 5% of vitaminized starch, both described by Mitchell et al. ('50), 2% of fiber (wood flock), 1% of sodium chloride, 0.5% of wheat germ oil, 1.5% of cod liver oil, 10% of sucrose, 10% of lard minus the amount of residual oil in the egg preparation, and enough of an equal mixture of glucose and dextrin to complete 100%. By actual analysis the diets contained 8.06, 9.81, 11.88, 13.81, 15.69, 17.50 and 19.75% of protein, and 4.28, 4.34, 4.39, 4.41. 4.45, 4.50 and 4.52 cal. of gross energy (heat of combustion) per gram. The increasing heat of combustion with increasing protein content is due to the greater heat of combustion of

protein than of starch and dextrin, although the metabolizable energy or fuel value would not be appreciably affected by this substitution.

The feeding experiments were carried out on male albino rats with initial weights, adjusted by proper pre-feeding, in all cases within 3 gm of 50 gm and in most cases within 2 gm. The rats were fed in 6 units of 7 rats each, one rat on each of the experimental diets. Each unit was fed until the faster growing rat had gained approximately 100 gm or somewhat more in body weight. The units were fed as follows:

Unit 1. The food intake was equalized, as far as feasible, for all 7 rats at a level determined by the poorest eater in the unit. However, since maximum food intake was desired, the rats on the lowest and the highest levels of protein, which proved to be the poorest eaters, were not allowed drastically to hold back the food consumption of the other 5 rats.

Unit 2. The food intake was equalized for all 7 rats at a level equal approximately to three-fourths that of unit 1.

Unit 3. The food intake was equalized for all 7 rats at a level amounting to 54% that of unit 1.

Unit 4. The food intake was equalized for all 7 rats at a level amounting to 46% that of unit 1.

Unit 5. These 7 rats were fed ad libitum.

Unit 6. These 7 rats were also fed ad libitum.

At the end of their respective feeding periods the rats were killed with ether, the alimentary tracts washed out and the whole carcasses frozen solid, ground to a homogeneous mass and analyzed for nitrogen by the Kjeldahl method, using mercury as a catalyst, and the heat of combustion was determined in the Parr adiabatic oxygen bomb.

EXPERIMENTAL RESULTS

The relationship between average daily gain in body weight and the percentage of protein in the diet for the first 4 units in the experiment is shown graphically in figure 1. Inspection of this graph shows clearly, especially for units 1, 2 and 4, that the rate of growth increased with in-

creasing protein concentration in the diet until a level of 12% was reached, after which no further consistent increase in growth resulted. The relationship up to 12% seems to be a linear one; hence, for each unit a straight line has been drawn describing the relationship. At 12% and beyond, the relationship is well described by a straight line of zero slope

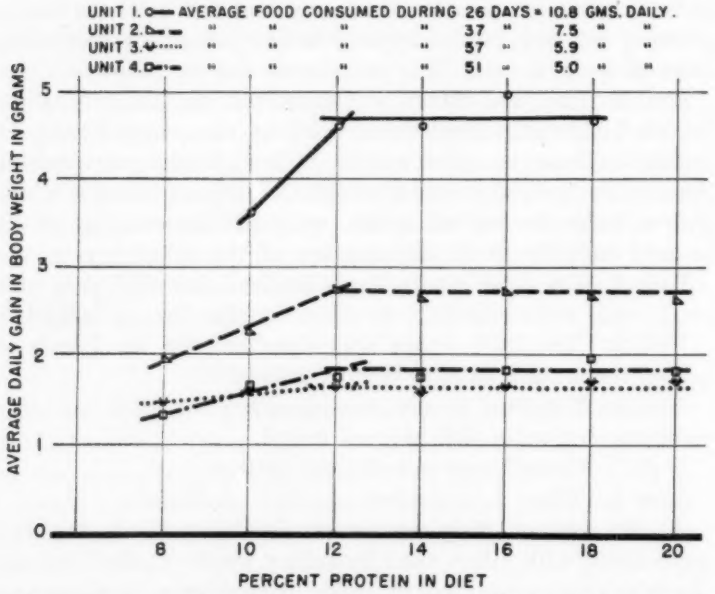

Fig. 1 The relationship between the per cent protein in the diet and the average daily gain in body weight for units 1 to 4, fed equal amounts of food within each unit.

drawn at a height above the x axis equal to the mean average daily gain of the 4 to 5 rats involved.

There is no indication from this figure that change in food intake and in resulting rate of growth among the different units affected appreciably the indicated percentage dietary protein required for maximum growth. The less clear-cut verdict rendered at the two lower levels of food consumption

would quite probably have been clarified if the experiments had been carried for a longer period. This question has been discussed by Watkins and Mitchell ('36) and justification for the assumption has been presented by Mitchell ('35), who showed in paired-feeding experiments that a cystine deficiency in casein could be demonstrated in one week of feeding when the experiment was conducted in the usual fashion; however, if the food intake of all pairs was deliberately and severely restricted, the demonstration of a cystine deficiency was not realized until three weeks of feeding had elapsed.

The relationship between protein level in the diet and the resulting daily gains in units 5 and 6, which were fed ad libitum, is shown in figure 2. The relationship is more irregular, as might be expected from the failure to equalize food consumption. In unit 5 the critical protein level seems clearly established at 12%; for unit 6 it may be taken at either 10 or 12%. But with larger food intakes and more rapid growth than the other units, there is no clear tendency for the protein requirement for maximum growth to shift to levels higher than 12%, the required level deduced from the response of the units on controlled and restricted feeding.

Also, in the controlled feeding units in these experiments the variation in body weight gains for those rats receiving an adequate protein diet, i.e., one containing 12 to 20% of protein, was smaller than in the ad libitum feeding units. This may be measured by the standard deviations, which were, for units 1 to 4, 0.162, 0.051, 0.063 and 0.090 gm per day; for units 5 and 6 fed ad libitum the standard deviations were 0.292 and 0.314 gm per day. The greater precision resulting from control of the food consumption of experimental animals has been noted by Shrewsbury and Bratzler ('33).

The analysis of the carcasses of the experimental rats for nitrogen and for heat of combustion has been used in estimating the daily deposition of nitrogen and energy during the feeding periods. The initial content of the rats in protein ($N \times 6.25$) and in energy was assumed to be 10 gm

and 100 cal., respectively, based on the average initial body weight of 50 gm and an average percentage of protein of 20% and an average energy value of 2 cal. per gram; the selected protein content was somewhat more than the observed aver-

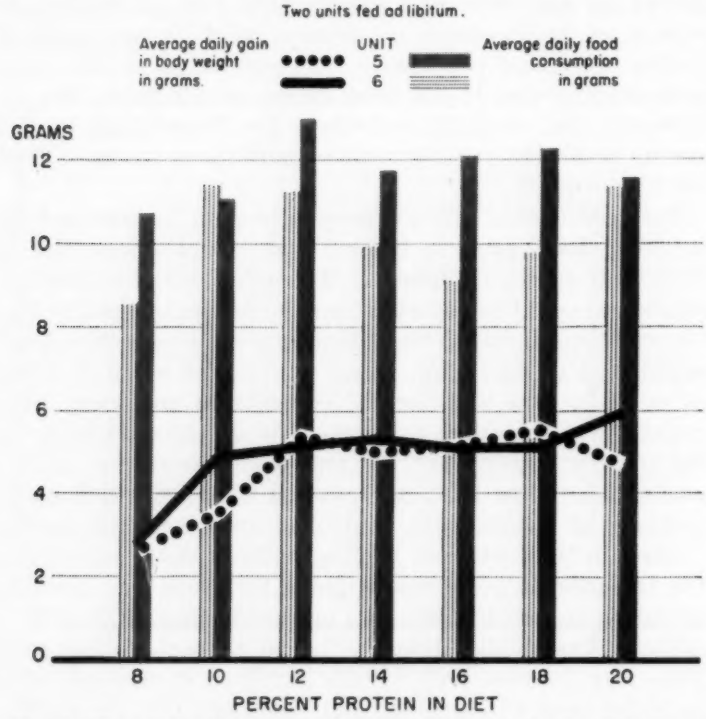

Fig. 2 The relationship between the per cent protein in the diet, the average gain in body weight and the average daily food consumption for units 5 and 6 fed ad libitum.

age at the termination of the feeding periods, i.e., 19.50%, and the energy content was somewhat less than the average terminal value of 2.14 cal. per gram.

The estimates of the daily deposition of protein and of energy for the 6 experimental units are assembled in table 1. The values for the daily protein deposition with increasing

TABLE 1

Daily deposition of protein and of energy by the experimental rats

UNIT NO.	DAILY FOOD CONSUMED	DAYS ON EXPERIMENT	DAILY DEPOSITION	PERCENTAGE OF PROTEIN IN EXPERIMENTAL DIETS						
				8	10	12	14	16	18	20
	gm									
1	10.8	26	Protein, gm	...	0.607 [1]	0.803	0.816	0.935	0.800	...
			Energy, cal.	...	9.81	10.08	10.81	8.69	11.23	...
			Cal. per gm protein	...	16.2	12.6	13.2	9.3	14.0	...
2	7.5	37	Protein, gm	0.331	0.416	0.522	0.525	0.508	0.521	0.514
			Energy, cal.	4.11	4.92	4.76	4.84	5.11	5.70	4.46
			Cal. per gm protein	12.4	11.8	9.1	9.2	10.1	10.9	12.6
3	5.9	57	Protein, gm	0.274	0.286	0.303	0.302	0.312	0.321	0.340
			Energy, cal.	3.60	3.77	3.81	3.42	3.33	3.51	3.37
			Cal. per gm protein	13.1	13.2	12.6	11.3	10.7	11.0	9.9
4	5.0	51	Protein, gm	0.207	0.272	0.299	0.311	0.331	0.356	0.324
			Energy, cal.	3.27	3.78	3.69	3.39	3.82	3.90	3.71
			Cal. per gm protein	15.8	13.9	12.3	10.9	11.5	11.0	11.4
5	ad lib.[2]	24	Protein, gm	0.490	0.670	0.970	0.994	0.943	1.063	0.942
			Energy, cal.	5.33	6.96	12.17	10.13	9.63	9.58	8.75
			Cal. per gm protein	10.9	10.4	12.6	10.2	10.2	8.5	9.3
6	ad lib.[3]	21	Protein, gm	0.417	0.724	0.798	0.893	0.827	0.783	0.930
			Energy, cal.	5.90	10.10	12.05	10.10	7.81	8.62	9.52
			Cal. per gm protein	14.1	13.9	15.1	11.3	9.4	11.0	10.2

[1] The average daily food consumption was somewhat less than that for its unit, namely, 10.5 gm.
[2] The average daily food consumption on rations containing 12% or more of protein was 12.2 gm.
[3] The average daily food consumption on rations containing 12% or more of protein was 10.4 gm.

levels of protein in the diet confirm the body weight gains in establishing the required protein level at 12% for units 1, 2 and 5. Unit 3 may be interpreted in the same sense if the values for the two highest protein levels, 0.321 and 0.340 gm per day, may be considered as of the same population as those for the 12, 14 and 16% levels, i.e., 0.303, 0.302 and 0.312 gm per day. The results for units 4 and 6 are not readily interpretable, either because no clear break in the upward trend of the value is evident with increasing protein levels, as in unit 4, or because the values are so irregular as to defy precise interpretation, as in unit 6.

The average daily deposition of energy showed no obvious correlation with the protein level in the diet in any of the 6 units of the experiment. However, the ratio of energy deposited to protein deposited in the body of the rats showed a distinct tendency among the units involving controlled feeding, exemplified best in unit 2, to be high at low protein levels, minimum at intermediate protein levels, and high again at the highest protein levels. This finding is in agreement with the conclusion of Mitchell and Hamilton ('35) that the ratio of fat to protein deposited in the bodies of growing animals (pigs) is higher on diets deficient in protein than on diets adequate in protein. The tendency in this experiment for the ratio of calories deposited to protein deposited to increase at the higher protein levels, particularly evident in unit 2, may mean a modification in metabolism leading to an increased conversion of glucose to fat.

DISCUSSION

The most significant finding of these experiments is the fact that the indicated minimum level of dietary protein required for the most rapid growth is not a function of the rate of food consumption attained in experimental feeding tests, within wide limits. It can be determined more clearly under conditions of controlled food consumption with its associated restricted growth than under conditions of ad libitum feeding. Specifically, a reduction in the average daily

intake of food beyond the indicated required protein level of 12%, from 12.2 gm (unit 5) to 7.5 gm (unit 2), or even to 5.9 gm (unit 3), did not alter the indicated protein requirement expressed as a percentage of the total feed. Furthermore, the variation in rate of gain beyond the 12% protein level was much less in the controlled units than in the units fed ad libitum.

The finding that the indicated protein requirement for maximum growth is independent of the rate of growth attained in controlled feeding experiments does not mean that rapid growth requires no greater concentration of protein in the diet than slow growth. Rather, it means that, when protein is the only factor in a diet limiting the growth of an animal, then, regardless of the rate of growth realized, a supplement of protein will induce a more rapid rate of growth on the same amount of food until the diet contains a level of protein adequate for maximum growth.

The proposition can be stated in a more general way as follows: If equal amounts of two diets, equally adequate for the animal functions under study except for the concentration of some one essential nutrient, are fed to two comparable animals, the better diet nutritionally with reference to this essential nutrient will always promote a better nutritional condition. If the essential nutrient is intimately involved in the processes of growth, such as protein, a more rapid growth will be associated with the greater concentration of the nutrient. If the essential nutrient is not intimately involved in the processes of growth, such as choline, then the better nutritive condition induced by the better-balanced diet will be revealed only on examination of those tissues in the functioning of which the nutrient is intimately involved, such as the liver and kidneys in the case of choline (Neumann et al., '49). If the concentration of the essential nutrient in the two diets under comparison is sufficiently high in both cases to promote maximum performance in the experimental animals, no appreciable difference in growth or tissue composition will occur on equicaloric intakes — as was the case in

unit 2, for example, with dietary protein levels of 12% or greater — until a dietary concentration of the essential nutrient is reached that exerts a harmful physiological effect.

In ad libitum feeding comparable rations are not fed in equal amounts to comparable animals in assessing nutritive requirements quantitatively for growth or other purposes. However, if it may be assumed that, through some unknown and undemonstrated physiological mechanism, the nutritionally better of two comparable diets will *always* be consumed in the greater amount, then the nutritive superiority of one of the two diets will be revealed by greater growth or more nearly normal tissue composition, or both, again regardless of the particular rate of growth (or other performance) that happens to have been attained.

It is fortunate indeed for nutritional research that nutritive requirements for maximum growth (or other function) can be assessed without realizing maximum performance in actual experimental studies. In rat studies, for example, few investigators have realized the rapid growth rates reported by Anderson and Smith ('32). It seems likely that these high growth rates resulted from pampering the appetites of the animals by the type of dietary components used, rather than by providing dietary nutrients essential for growth in proportions "optimal for the growth of the body, organs and body components" as Dunn, Murphy and Rockland ('47) have concluded. Synthetic diets, so effective in modern nutritional research, are so often unacceptable to experimental animals that, unless force-fed, they do not support maximum physiological performance. Nevertheless, nutrient requirements determined with such diets are not discounted, and rightly so, because of failure to secure the consumption of food that would be realized on diets containing a judicious selection of natural foods. A prominent example of this sort is the work of Rose ('37, and subsequent papers) on the amino acid requirements for growth in the rat. The published values expressed as percentages of the diet are described as requirements for "normal" or "optimum"

growth, although the rates of growth actually realized were distinctly submaximum (Womack and Rose, '46) averaging only 3.5 gm daily. Nevertheless, these values are cited, as they probably should be, without reference to the slow rates of growth with which they are associated. The conclusion of Williams and Spies that "all species of animals require somewhat less than 1 part per million of thiamine in the food when carbohydrate predominates in the diet to the degree which is usual for most species," is cited with approval by Arnold and Elvehjem ('39), with no qualifications concerning the rates of growth experimentally realized in the basic research. The same situation has been pointed out by Mitchell et al. ('50) with reference to riboflavin.

The indicated requirement of 12% of whole egg protein in a diet containing 12% fat relates to the body weight interval of from 50 gm to approximately 150 gm or somewhat more. It is lower than the value of 16% obtained by Hamilton ('39) with commercial, rather than laboratory-prepared, whole egg protein. The necessary protein level for maximum growth will vary with the stage of growth: thus, growing pigs require of a given protein mixture more than 26% of the diet at a weight of 40 to 50 lb. for maximum nitrogen retention, about 22% at 100 lb., about 17% at 150 lb., and about 15% at 175 to 200 lb. (Mitchell et al., '36-'37). However, at any given body weight for any species of animal the necessary percentage of a given protein in a diet of definite caloric density may be considered to be a definite value, regardless of the amount of food consumed, in the sense that at any level of food intake a higher proportion of the protein induces no greater growth. This conception is supported by the findings of the present investigation, but is contrary to that of Crampton ('40), who believes "that protein requirements determined by any method are inseparably linked with average daily food consumption . . ." The present authors venture the opinion that the relationship of body weight gain to protein consumption at any level of food intake cannot proceed indefinitely along a linear regression line.

CONCLUSIONS

1. In the growing rat, the protein percentage in diets of equal caloric density necessary for maximum growth at any caloric intake level is independent of the caloric intake level within a considerable range. For the diets employed in this investigation, for the segment of the growth curve traversed and for the dietary protein studied (whole egg protein prepared in the laboratory at temperatures not exceeding 100°C.) this percentage is approximately 12.

2. This necessary protein level in the diet can be more precisely determined under conditions of controlled feeding than of ad libitum feeding if the food intake is such as to promote a certain minimum rate of gain, equal in the rat to about 2 gm daily.

3. When protein is the only factor in a diet limiting the growth of an animal, then regardless of the rate of growth realized, a supplement of protein will induce a more rapid rate of growth on the same amount of food.

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IMPROVING THE NUTRITIVE VALUE OF FLOUR

V. THE EFFECT OF THE USE OF DEFATTED WHEAT GERM

BEULAH D. WESTERMAN, FAITH ROACH AND MIRIAM STONE[1]
Department of Foods and Nutrition, Kansas Agricultural Experiment Station, Kansas State College, Manhattan

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Several investigators have shown that enriching flour with thiamine, riboflavin, niacin, and iron is beneficial in promoting better nutrition (Westerman, Linn, Templeton and Wells, '49; Goldsmith, Darby, Steinkamp, Beam and McDevitt, '50; Goldsmith, '50; Westerman and Stone, '51). However, there may be other factors involved besides the B vitamins and iron which are added to the flour and bread in the enriching process. Wheat germ is one of the products removed when flour is milled and this substance contains many vitamins and minerals, as well as protein. After the removal of the oil, the wheat germ may be ground to a fine powder which mixes readily with the flour and has better keeping qualities than when the fat is present. On the basis of these facts it was decided to study the nutritive quality of defatted wheat germ when used with enriched and non-enriched flour. The experiments were conducted with albino rats and three methods of evaluation were used: the growth test, performance during pregnancy and lactation, and storage of the vitamins in the liver.

PROCEDURE

Foods similar to those consumed by human beings were used in the diets. Enriched or non-enriched flour furnished

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48% of the total calories, with sugar, fat, meat, dried milk, eggs, legumes, potatoes, carrots, green vegetables and apples making up the remainder. The exact composition of the diets and the method of preparation are described fully in previous publications (Westerman et al., '49; Westerman and Stone, '51).

The diets for all the groups were the same except as follows: diet I contained enriched flour; diet II non-enriched flour; diet III enriched flour with 2% wheat germ added; diet IV non-enriched flour with 2% wheat germ; diet V enriched flour and 4% wheat germ; diet VI non-enriched flour and 4% wheat germ; diet VII enriched flour and 6% wheat germ; and diet VIII non-enriched flour and 6% wheat germ. The wheat germ was added at the 2% level, as that is the amount found in the whole wheat grain, and the other levels were chosen to test whether larger quantities might have additional beneficial effects.

Albino rats weighing from 45 to 50 gm and 28 days of age were placed on a B-complex-free diet for two weeks to deplete their tissues of the stored vitamins. At the end of the depletion period the animals were divided into groups of 8 females and 4 males for diets I through IV and 6 females and 4 males for diets V through VIII. There was equal distribution in regard to weight and litter mates. This particular distribution regarding sex was made so that reproduction as well as growth could be evaluated. The animals were weighed at weekly intervals for a period of 10 weeks. At the end of the growth test, the animals were bred and the females were carried through three reproduction and lactation periods. Then they were sacrificed and the livers taken for vitamin analysis. The livers of two animals were combined to provide samples large enough for these determinations. In order to have the data comparable, the results are reported on a dry fat-free basis. The fat was determined according to the method of the Association of Official Agricultural Chemists ('45) and the moisture by the method recommended for the co-operative meat investigations (State

Agricultural Experiment Stations and U. S. Department of Agriculture, '44). The thiochrome procedure of Hennessy ('41) was used for thiamine, the fluorometric method of Peterson, Brady and Shaw ('43) for riboflavin, and the microbiological methods of Strong, Feeney and Earle ('41) and Krehl, Strong and Elvehjem ('43) for pantothenic acid and niacin.

RESULTS AND DISCUSSION

Calculations of the nutritive value of the diets based on the daily food consumption of human beings are shown in table 1. The addition of wheat germ increased the amount of protein, iron, thiamine, riboflavin and niacin in the diets, and perhaps added other essentials. Analyzing the diets from the point of view of human needs, they were fairly adequate in protein but provided only about 50% of the calcium requirement. The iron was adequate in those diets containing enriched flour but inadequate when non-enriched flour was included. A larger proportion of the calories was from carbohydrate than from fat, which should increase the need for the B vitamins. The diets containing non-enriched flour were below adequacy in riboflavin and niacin, while the amount of thiamine seemed to be adequate in all the diets.

The average weight gains of the rats during the 10-week growth test are shown in table 2. In comparing these gains, it may be noted that those animals on diets I, III, V, and VII containing enriched flour made greater weight gains than those on the other diets which contained non-enriched flour. The addition of wheat germ to the enriched flour aided in increasing the growth rate, when added at the 4 and 6% levels. However, addition at the 6% level did not increase the rate of growth over that obtained with the 4% level, diets VII and V. The addition of 2% wheat germ to enriched flour did not increase the growth rate of the animals above that obtained with enriched flour alone, diets I and III, but had considerable effect upon the growth rate when the diets contained non-enriched flour, diet II as compared to diets IV, VI and VIII.

TABLE 1

Nutritive value of diets based on the daily food consumption of some human beings

	CAL.	PROTEIN	FAT	CHO	Ca	Fe	VIT. A	THIAMINE	RIBOFLAVIN	NIACIN	VIT. C
		gm	*gm*	*gm*	*gm*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
Diet I, enriched flour	2,254	61.4	69.40	430	0.50	14.9	10,093	3.11	1.65	19.7	80
Diet II, non-enriched flour	2,254	61.4	69.40	430	0.50	7.9	10,093	1.12	0.89	7.4	80
Nutrients added by 2% wheat germ	22	2.4	0.01	3	0.01	0.6		0.21	0.05	0.4	

TABLE 2

Comparison of means of total weight gains by sex and diets[1]

	I ENRICHED FLOUR	II NON-ENRICHED FLOUR	III ENRICHED + 2% WHEAT GERM	IV NON-ENRICHED + 2% WHEAT GERM	V ENRICHED + 4% WHEAT GERM	VI NON-ENRICHED + 4% WHEAT GERM	VII ENRICHED + 6% WHEAT GERM	VIII NON-ENRICHED + 6% WHEAT GERM
♀	131.3	121.2	137.0	134.2	163.0	133.3	163.5	141.3
♂	206.0	164.2	204.2	184.0	232.0	218.2	236.0	220.5
Diet	161.2	138.4	163.9	154.1	190.6	167.6	192.5	173.0

[1] Test of significance: I > II at 0.1% level for males; non-significant for females. III > IV at 1.0% level for males; non-significant for females. V > VI at 5.0% level for males and at 0.1% level for females. VII > VIII at 1.0% level for both sexes. V > I and VII > I at the 0.1% level for both sexes. IV > II at 1.0% level for males; non-significant for females. VI > II at 0.1% level for males; non-significant for females. VIII > II at 1.0% level for females and at the 0.1% level for males.

The data were analyzed for statistical significance by the "t" test. Considerable interaction between the sexes was found, therefore the test for significance was determined for each sex. The males were sensitive to differences between the enriched and non-enriched flour without wheat germ or with 2% wheat germ added, whereas the females were not. Male rats on diet I showed a significantly greater weight gain than those on diet II, at the 0.1% level; also those on diet III showed a significantly greater gain over those on diet IV at the 1% level; but the differences were non-significant for the females in those groups. When 4 or 6% wheat germ was added, both sexes were sensitive to the differences between enriched and non-enriched flour. Animals on diet V showed significant differences over diet VI at the 0.1% level for the females and for the males at the 5% level. Significant differences at the 1% level were shown by animals on diet VII over those on diet VIII for both sexes.

When enriched flour was used, the sexes were about equally sensitive to the addition of wheat germ, as shown by the significantly greater gains at the 0.1% level for both sexes, diets V and VII, over those obtained on diet I. There were no significant differences shown for either sex between diets I and III.

With the non-enriched flour, the males again were more sensitive to the addition of wheat germ than were the females, as the males on diet IV showed weight increases which were significant at the 1% level and on diet VI at the 0.1% level over those on diet II. The differences between the females on these diets were non-significant but were near significance and showed the same trends as those of the males. When 6% wheat germ was added, as in diet VIII, the females showed significant differences at the 1% level and the males at the 0.1% level over diet II, containing non-enriched flour without wheat germ.

These data indicate that the addition of 2, 4 or 6% wheat germ to non-enriched flour aided in promoting growth, while

enriched flour required the addition of 4 or 6% wheat germ to increase the growth rate over that produced by enriched flour alone in the diet. Wheat germ at the 4% level seemed to be adequate, as 6% wheat germ did not cause a greater growth increase.

A summary of the reproduction and lactation performance is shown in table 3. These data show the same trend as the growth data. The females on diets I and III containing enriched flour and enriched flour plus 2% wheat germ produced 63% of the total possible litters, while those on diet II with non-enriched flour produced 57% of the total litters possible. Of these, 43% survived on diet I and 42 and 29% on diets II and III, respectively. Those on diet IV with non-enriched flour and 2% wheat germ had 55% of the possible litters and raised 42% of these. The highest percentage, 87% of the possible litters, was produced by the females on diet V, containing enriched flour with 4% wheat germ, and 69% of these survived. Animals on diet VII, containing enriched flour and 6% wheat germ, produced 71% of the total possible litters, and 70% of the litters survived. Those on diets VI and VIII, containing non-enriched flour and 4 and 6% wheat germ, respectively, produced 79 and 56% of the total number of litters, and 55 and 60%, respectively, of these survived.

The average birth weight of the young rats and the average weight at the end of 28 days were higher among those on the rations containing 4 and 6% wheat germ; diets V, VI, VII and VIII. These values for the animals on diets V and VII containing enriched flour were greater than those on diets VI and VIII, with non-enriched flour. Here again the enriched flour with 4% wheat germ seemed to give about the same results as 6% wheat germ.

The data on the amount of thiamine, riboflavin, pantothenic acid and niacin found in the livers of the rats are shown in table 4. Data from diets III and IV containing 2% wheat germ were not available. For purposes of comparison, analyses were also made of the livers of rats from the stock

TABLE 3

Summary of reproduction and lactation performance

	DIET	NO. OF FEMALES	REPRODUCTION PERIOD	NO. OF LITTERS	AVE. NO. LIVE YOUNG PER LITTER	AVE. WT. AT BIRTH	NO. OF LITTERS SURVIVING	AVE. NO. WEANED PER LITTER	AVE. WT. 28 DAYS
I.	Enriched flour	8	1st	6	5.5	5.7	1	4	36
		7	2nd	2	7.5	5.5	0		
		7	3rd	6	6.6	6.2	5	4	42
II.	Non-enriched flour	7	1st	5	6.0	5.0	3	3	42
		7	2nd	2	5.5	7.0	0		
		7	3rd	5	5.5	6.0	2	7.5	39
III.	Enriched + 2% wheat germ	8	1st	6	6.3	4.6	1	3	35
		7	2nd	2	4.5	4.7	1	3	31
		7	3rd	6	5.8	6.0	2	7	45
IV.	Non-enriched + 2% wheat germ	8	1st	4	2.0	5.0	0		
		7	2nd	2	2.0	4.0	0		
		7	3rd	6	8.3	6.0	5	6	53
V.	Enriched + 4% wheat germ	5	1st	4	7.5	6.4	2	7.5	44
		5	2nd	5	9.0	6.4	4	8.7	50
		5	3rd	4	9.7	6.2	3	9.3	50
VI.	Non-enriched + 4% wheat germ	5	1st	3	9.6	4.5	0		
		5	2nd	4	7.0	5.7	3	6.3	41
		4	3rd	4	5.5	6.0	3	5.3	55
VII.	Enriched + 6% wheat germ	5	1st	1	10.0	5.2	0		
		5	2nd	5	8.2	6.1	3	7.3	56
		4	3rd	4	10.0	6.0	4	9.5	51
VIII.	Non-enriched + 6% wheat germ	6	1st	2	4.0	5.0	0		
		6	2nd	5	7.5	6.3	3	7.0	49
		6	3rd	3	9.0	6.3	3	5.6	50

colony. The "t" test of statistical significance was applied to the data. The livers of the rats on diet I, with enriched flour, showed a greater content of thiamine than those of animals on diet II with non-enriched flour, and the difference was significant at the 1% level. The differences among the

TABLE 4

Average vitamin content of rat livers [1]

DIET NO.	DESCRIPTION	THIAMINE	RIBOFLAVIN	PANTOTHENIC ACID	NIACIN
		μg/gm	*μg/gm*	*μg/gm*	*μg/gm*
I.	Enriched flour	19.5	59.3	50.9	404.5
II.	Non-enriched flour	7.7	39.1	81.2	528.3
V.	Enriched + 4% wheat germ	13.3	64.6	109.1	475.5
VI.	Non-enriched + 4% wheat germ	10.3	55.9	132.8	453.0
VII.	Enriched + 6% wheat germ	16.8	62.1	74.4	430.8
VIII.	Non-enriched + 6% wheat germ	9.6	50.1	123.9	602.1
	Stock diet	19.9	65.0	70.4	375.9

Test of significance on vitamin content of rat livers

MEAN SQUARES

Sources of variation	D/F	Thiamine	Riboflavin	Pantothenic acid	Niacin
Diet	5	70.53 [2]	293.86	3770.3 [3]	16,820
Samples	17	15.13	215.07	1108.9	13,093
Total	22				

[1] Test of significance: I > II at the 1% level for thiamine, differences in other vitamins were non-significant. I > V at the 5% level for thiamine; V > I at the 5% level for pantothenic acid. VI > II at the 5% level for pantothenic acid; non-significant differences for the other vitamins. Non-significant differences between I and VIII; II and VIII; V and VI. VII > VIII at the 5% level for thiamine only.

[2] Significant at 1% level.

[3] Significant at 5% level.

other vitamins were non-significant. Also, the thiamine content was significantly greater at the 5% level for those rats on diet I than for animals on diet V, but those on diet V had significantly higher pantothenic acid content at the 5% level than those on diet I. It is likely that the 4% wheat germ added to the enriched flour supplied more pantothenic acid than the enriched flour alone and allowed greater storage, particularly if the storage capacity had not been fully used. This is shown again by the animals on diet VI, containing 4% wheat germ added to non-enriched flour, whose livers contained significantly greater amounts, at the 5% level, of pantothenic acid than did those of the rats on diet II containing non-enriched flour alone. The differences for the other vitamins were non-significant. There were non-significant differences between the animals on diets I and VII; II and VIII; and V and VI for the 4 vitamins, while the rats on diet VII had a significantly larger amount, at the 5% level, of thiamine than did those on diet VIII. The average amount of thiamine found in the livers of the stock animals and of those with enriched flour in the diet was about the same, 19.5 μg/gm, while those with enriched flour and 6% wheat germ averaged 16.8 μg/gm and those with 4% wheat germ 13.3 μg/gm. If the stock diet is considered adequate, allowing for maximum storage, and diet I allowed maximum storage, then it is difficult to explain why the diets with added wheat germ, V and VII, did not permit maximum storage, particularly when wheat germ contains 32.8 μg/gm of thiamine. The animals on all the diets containing non-enriched flour, even though wheat germ was added, did not store as much of this vitamin in the livers as did the stock animals.

The differences in the amounts of riboflavin and niacin in the livers of the rats kept on the different diets were not statistically significant, but it may be noted that those animals on diets containing non-enriched flour had a lesser amount of riboflavin in the liver than those on diets containing enriched flour; also, those on non-enriched flour without wheat germ had the least amount of this vitamin in the liver.

Wheat germ contains 7.8 μg/gm of riboflavin. These data demonstrate some benefits from the addition of riboflavin to the diet through the enriched flour and the wheat germ. The niacin content of the livers of the stock animals was less than that of the experimental animals, which would indicate that the experimental diets were adequate in this vitamin or its precursors.

The livers of those rats receiving wheat germ showed a larger storage of pantothenic acid than did those of the others. Wheat germ is a good source of pantothenic acid, containing 33.8 μg/gm of this vitamin, which evidently accounts for the greater amounts in the livers. Evidently the diets without wheat germ, including the stock diet, did not contain enough of this vitamin to promote maximum storage in the liver.

SUMMARY

Experiments have been conducted with the albino rat to determine the nutritive effect of the addition of wheat germ to enriched and non-enriched flour at the 2, 4, and 6% levels. The results of the growth test showed that the addition of wheat germ at the 2% level did not increase the growth rate above that obtained with enriched flour alone; at the 4 and 6% levels the growth rate was increased, but at the same rate for both levels. At all three levels, the addition of wheat germ to non-enriched flour produced an increase in growth.

The reproduction and lactation performance showed the same trends as the growth test. The females fed enriched flour and 4% wheat germ produced the largest number of litters and more of these litters survived.

The rat livers were analyzed for thiamine, riboflavin, pantothenic acid and niacin. Statistically significant differences were found in thiamine content, the animals with enriched flour in the diet having a higher content of thiamine than those with non-enriched flour. The differences between these two diets for the other vitamins were non-significant statistically. The animals fed enriched flour and wheat germ

in the diet stored more thiamine in the livers than did those fed non-enriched flour and wheat germ. The pantothenic acid in the wheat germ provided for much greater storage of this vitamin, as the rats with wheat germ in their diets had considerably more of this vitamin in their livers than did those without wheat germ.

The data indicate that the addition of defatted wheat germ to the flour produced an increase in growth rate and better reproduction and lactation performance, as well as an increased storage of some vitamins, particularly pantothenic acid, in the liver.

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